SPECIFICITY AND IMMUNOSUPPRESSION OF HUMAN CYTOTOXIC T LYMPHOCYTE RESPONSES TO HERPES SIMPLEX VIRUS

by

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ABSTRACT

Cytotoxic T lymphocytes (CTL) are important in controlling a number of viral infections including those caused by members of the herpesvirus family. Many studies have focussed on the phenotype of human CTL specific for herpes simplex virus (HSV) although few studies address their specificity. HSV glycoproteins B (gB) and D (gD) can serve as target antigens for $CD4^+$ and $CD8^+$ anti-HSV CTL clones generated by repeated stimulation of peripheral blood mononuclear cells (PBMC) with HSV antigens or HSV-infected cells. In the present study, the presence of gB- and gD-specific CTL in polyclonal cultures of anti-HSV CTL stimulated with a single in vitro exposure to HSV-1 was examined using autologous EBV-transformed B-lymphoblastoid cell lines (LCL) infected with recombinant vaccinia virus or adenovirus vectors encoding gB (vgB11, AdgB) or gD (vgD52, AdgD). Of six HSV seropositive donors tested, only one individual generated $CD4^+$ and $CD8^+$ T cells capable of lysing LCL infected with HSV, vgB11 or vgD52. Anti-HSV CTL from the remaining five HSV seropositive donors lysed HSV-infected LCL only. Therefore, gB and gD can serve as target antigens for polyclonal cultures of human anti-HSV CTL although the majority of the bulk CTL response in most patients is directed at HSV antigens other than gB and gD. LCL infected with recombinant adenoviruses were not recognized by gB- and gD-specific CTL due to the restricted expression of the inserted gene product.

Since LCL infected with recombinant adenoviruses were not lysed by human anti-HSV CTL, human fibroblasts, which are permissive to adenovirus infection,

were chosen as potential target cells. However, human fibroblasts infected with HSV-1 (HSV-FB) were not lysed by human anti-HSV CTL or human allo-antigen specific CTL (allo-CTL). HSV-FB were not only resistant to lysis by human CTL, but exposure of CTL to HSV-FB rendered the CTL unable to lyse its normally sensitive target cell. Studies concerning the mechanism of this inhibition suggested that there were two distinct mechanisms of inhibition of CTL lysis. The first involved FB infected with HSV-1 for 2 hours and required the expression of ICP4, an immediate-early protein of HSV-1, but not infectious virus or virus-induced shut-off of host protein synthesis. The second mechanism of inhibition occurred later in the HSV replication cycle and involved the infection of CTL via cell-to-cell spread of HSV-1 from FB infected for 20 hours. The elucidation of mechanisms involved in HSV-induced immunosuppression may foster the development of preventative or therapeutic strategies aimed at controlling these pathogens in humans.

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PREFACE

The data in this thesis are presented in four chapters. Each chapter represents a manuscript that has been published or submitted to a peer-reviewed journal. *Chapter 2*, entitled "Analysis of Human Anti-Herpes Simplex Virus Cytotoxic T Lymphocytes Specific for Glycoproteins B and D in Polyclonal Cultures", has been submitted as a short communication to *Viral Immunology* and studies the prevalence and phenotype of gB- and gD-specific CTL in six HSV-seropositive donors. *Chapter 3*, entitled "Restricted Infection of Human B Lymphoblastoid Cells by Recombinant Adenoviruses: Inability to Serve as Cytotoxic T Lymphocyte Targets", has been submitted to the *Journal of General Virology* and focusses on the use of recombinant adenoviruses in analysing the specificity of human anti-HSV CTL when EBV-transformed B lymphoblastoid cell lines are used as target cells. *Chapter 4*, entitled "Herpes Simplex Virus-Infected Human Fibroblasts are Resistant to and Inhibit Cytotoxic T Lymphocyte Activity", was published in the *Journal of Virology* (66:6264-6272, 1992) and characterizes the resistance of HSV-infected fibroblasts to human anti-HSV CTL and allo-antigen specific CTL lysis. *Chapter 5*, entitled "Inhibition of Human CTL-Mediated Lysis by Fibroblasts Infected with Herpes Simplex Virus", has been accepted to *The Journal of Immunology* for publication and studies the mechanism of inhibition of CTL lytic function by HSV-infected fibroblasts. The chapters are preceeded by *Chapter 1*, an introduction to the role of the immune system in herpesvirus infections. The chapters are followed by *Chapter 6*, a discussion unifying the

concepts and issues raised in the papers. This format has been approved by the supervisory committee for Christine M. Posavad and by the School of Graduate Studies at McMaster University.

The thesis candidate wrote the four scientific manuscripts with editing assistance from the co-authors. Dr. David C. Johnson co-authored *Chapter 3* because of the use of the recombinant adenovirus vectors contructed in his lab and his invaluable advice regarding expression of recombinant adenoviral insert genes. Dr. Fiona M. Smaill was also included as a co-author because she performed the studies involving the Ad5LacZ vector. Ms. Jennifer M. Newton was a co-author of *Chapter 5* because she provided essential technical assistance in running the immunoprecipitation and SDS-PAGE and in the sandwich assays.

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LIST OF ABBREVIATIONS

ADCC	antibody-dependent cellular cytotoxicity
AIDS	acquired immunodeficiency syndrome
allo-CTL	alloantigen-specific cytotoxic T lymphocyte
CMV	cytomegalovirus
CTL	cytotoxic T lymphocyte
EBV	Epstein-Barr virus
FcR	Fc receptor
FACS	fluorescence-activated cell sorter
FB	fibroblast
HHV-6	human herpesvirus type 6
HIV	human immunodeficiency virus
HSV-1	herpes simplex virus type 1
HSV-2	herpes simplex virus type 2
HSV-FB	HSV-infected fibroblast
LAK cells	lymphokine-activated killer cells
LCL	EBV-transformed B lymphoblastoid cell line
MHC	major histocompatability complex
NK cell	natural killer cells
PBL	peripheral blood lymphocytes
PBMC	peripheral blood mononuclear cells
PHA	phytohemagglutinin

PrV	pseudorabies virus
TcR	T cell receptor
UV-HSV	UV-irradiated HSV
VZV	Varicella-zoster virus

CHAPTER 1

INTRODUCTION

A. HERPES SIMPLEX VIRUSES

A1. Herpesviridae family of viruses

Herpesviruses are large, double-stranded DNA viruses that are subdivided into three major subfamilies, alpha, beta and gamma depending on a number of in vivo and in vitro characteristics (reviewed in Roizman, 1982). Alpha herpesviridae remain latent in ganglionic tissue and are subdivided into 2 subgroups: alpha 1 herpesviruses are rapidly replicating viruses such as herpes simplex viruses type 1 (HSV-1) and type 2 (HSV-2) whereas alpha 2 herpesviruses replicate more slowly and include varicella zoster virus (VZV). Beta herpesviruses include the very slow replicating cytomegalovirus (CMV) which remains latent within lymphocytes. Gamma herpesviruses represent lymphotropic viruses typified by Epstein-Barr virus (EBV) that have the ability to immortalize cells and cause lymphoproliferative disease in infected individuals. The hallmark of all members of the herpesviridae family of viruses is their ability to persist throughout the lifetime of their hosts in latent, or silent, form (Stevens, 1989). This non-infectious state can last for years or latent virus may reactivate to allow the production of infectious virus (Stevens, 1989).

A2. Epidemiology of HSV infections

Although HSV-1 and HSV-2 can each cause oral or genital lesions, HSV-1 primarily causes oral/facial lesions while HSV-2 is the leading cause of ulcerative lesions of the genital tract (Aurelian, 1990; Mertz, 1990). It is estimated that 5-9

million people per year consult for genital HSV infection in the US (Aurelian, 1990). The increase in the incidence of this sexually-transmitted disease in the last 20 years has resulted from the increased number of sexual partners, earlier sexual activity and the introduction of oral contraceptive pills (Mertz, 1990). In contrast, a decline in the acquisition of HSV-1 infection in childhood has occurred (Mertz, 1990).

Transmission of HSV-2 can occur in patients with symptomatic or asymptomatic HSV infections. Asymptomatic shedding of HSV-2 occurs most commonly from HSV-2 seropositive persons with no clinical history of genital HSV disease (Mertz, 1990). Transmission of virus from a person with genital herpes was more frequent when the partner at risk lacked HSV-1 antibodies (Mertz, 1990). As for perinatal transmission of HSV, the virus infects nearly 50% of infants whose mothers had primary genital herpes whereas less than 5% of infants exposed to recurrent maternal infection at the time of delivery become infected (Arvin, 1991). Genital HSV infections have also been associated with an increased transmission of human immunodeficiency virus (HIV), the etiologic agent of acquired immunodeficiency syndrome (AIDS) (Mertz, 1990).

A3. Infection, Latency and Reactivation of HSV

The HSV virion is composed of an HSV genome packaged within a protein snell, or capsid, with an outer lipid envelope (reviewed in Roizman and Sears, 1990). The HSV genome is a linear, double-stranded DNA molecule (molecular weight approximately 100×10^6) with the capacity to encode over 70 proteins. The

icosahedral capsid is composed of 162 capsomers which surrounds the electron-opaque core. The outer envelope contains HSV glycoproteins embedded in a cell-derived lipid membrane. Between the capsid and the envelope is the tegument that contains a number of virion proteins.

Exposure of HSV at mucosal surfaces or abraded skin allows the entry of virus and replication in cells in the epidermis and dermis. After binding and fusion of the virus with cellular membranes, nucleocapsid is released into the cytoplasm and is transported to the cell nucleus. The HSV genome enters the nucleus where the expression of viral genes occurs in a highly regulated fashion (Hay and Ruyechan, 1992). The α, or immediately early (IE), proteins are the first produced and do not require prior synthesis of a viral protein. A tegument protein, α-transinducing factor (α-TIF), and a cellular protein, OCT-1, are necessary for transactivation of α proteins. α proteins are responsible for the transactivation and/or transrepression of the remaining viral proteins. Early (E), or β, proteins are produced next and are involved primarily in nucleic acid metabolism. The late (L), or γ proteins, are produced after DNA synthesis and are primarily structural proteins including glycoproteins and capsid and tegument components. With the production of L proteins, nucleocapsids are assembled in the nucleus and envelopment occurs as the nucleocapsids bud through the nuclear membrane. The virions are released from the cell by secretion

through the Golgi apparatus. The HSV-1 lytic cycle is approximately 18 hours long and depending on the cell type, $5x10^4$ to $2x10^5$ virions per cell are produced.

HSV produced in epidermal and dermal cells then infects the innervating sensory nerves. These cells may become productively infected or virus replication can be interrupted for long periods of time producing a reservoir of latent virus in the nervous system where periodic reactivations can occur. The events involved in establishment and maintenance of the latent state are not clear (Stevens, 1989). Immunosuppression, UV-light and other stimuli have been implicated in reactivating latent HSV. Reactivation of latent HSV results in the transport of infectious virus from the sensory nerves to the body surface where replication can again occur in the epidermal and dermal cells to produce the characteristic vesicular lesion of recurrent HSV infections.

A4. The role of the immune system in controlling HSV infections

A4(i). Animal studies

Several animal models have been developed to study the role of the immune system in controlling HSV infections. Extensive research has focussed on immunity to HSV in mice. Both humoral and cellular immune mechanisms appear to be involved in control of murine HSV infections. HSV-specific antibodies can confer protection against subsequent challenge (reviewed in Corey and Spear, 1986). Antibodies directed against viral glycoproteins B, C, D, E, and G have provided protection against ganglionic latency or neurologic disease although the

protective function (neutralization, ADCC) of these antibodies in vivo is not known (Corey and Spear, 1986).

Adoptive transfer experiments have demonstrated a critical role for both $CD4^+$ and $CD8^+$ T cells in protection from lethal HSV infections (Nash et al., 1987). $CD4^+$ T cells were presumed to function in delayed-type hypersensitivity while $CD8^+$ T cells were thought to function as CTL (Martin and Rouse, 1990). In vitro studies indicate that $CD8^+$ anti-HSV CTL predominate when mice are immunized with HSV, although $CD4^+$ anti-HSV CTL have also been detected (Martin and Rouse, 1990). The specificity of anti-HSV CTL has depended on MHC haplotype and many viral proteins, including gB, gC, gD, ICP4 and ICP27, can serve as target antigens (Martin and Rouse, 1990).

The guinea pig model of HSV disease more closely reflects human disease. Female guinea pigs infected vaginally with HSV develop a limited infection, establish latency in local sensory ganglia and deveiop recurrent disease (Stanberry, 1992). Humoral immune studies have been well characterized but, until recently, the examination of T cell mediated immunity has not been possible. Although no model is completely satisfactory in reflecting human infections, much knowledge has been attained regarding basic immune control of mammalian HSV infections.

A4(ii) Human Studies

Due to ethical considerations, the involvement of immune mechanisms in the control of human HSV infections is largely circumstantial. Most patients with

primary HSV infections develop relatively mild and localized lesions (Corey and Spear, 1986). Severe HSV infections and disseminated disease occurs primarily in immunocompromised or immunologically immature individuals, observations implicating a major role of the immune system in controlling primary and recurrent HSV infections. Severe, and often life-threatening, HSV infections occur in immunologically depressed patients including cancer patients (Bustamante and Wade, 1991), cardiac transplant patients (Rand et al., 1976), heart-lung transplant patients (Smyth et al., 1990) and renal transplant patients (Pien et al., 1973). Disseminated HSV infections, sometimes leading to encephalitis and/or death, in immunologically immature neonates of mothers with primary HSV infection can occur unless treated with antiviral therapy (Arvin, 1991). A much lower transmission of HSV to neonates from mothers with recurrent genital herpes compared with mothers with primary HSV infection suggest that maternal immunity to HSV can decrease the risk of neonatal transmission (Arvin, 1991).

Although cell-mediated and humoral immune mechanisms appear to be involved in controlling primary HSV infections and reactivations, patients with defects in cell-mediated immunity experience more severe HSV infections than patients with deficits in humoral immunity (Corey and Spear, 1986). Recurrent HSV lesions occur in the presence of serum neutralizing antibody (Kohl, 1992); further, HSV-specific antibody levels do not change significantly during the course of disease (Vestey et al., 1990; Douglas and Couch, 1970). A number of studies, discussed in a later section, have detected changes in cell-mediated immune

responses at different stages of HSV disease. Herpesviruses, including HSV, are among the most common opportunistic infections observed in AIDS patients (Siegal et al., 1981; Eversole, 1992; Quinn, 1990) where HIV targets the $CD4^+$ T cell leading to its destruction. Taken together, these observations demonstrate a crucial role of cell-mediated immune regulatory functions in controlling HSV.

Non-specific anti-viral defence mechanisms, such as natural killer (NK) cells, macrophages and interferon production provide an early protective barrier to herpesviruses (Domke-Opitz and Zawatzkym, 1990). However, the control of infection and establishment of long-term immunity are mediated by the adaptive immune system, especially anti-viral cytotoxic T lymphocytes (CTL).

B. CTL

B1. Charactertics of CTL

CTL are important effector cells of the cell-mediated immune system that play a vital role in the resolution and control of many viral infections (Rouse et al., 1988). The α/β T cell receptor (TcR) on the surface of a CTL specifically recognizes peptide fragments of foreign antigen presented by self major histocompatability complex (MHC) antigens (reviewed in Brodsky and Guagliardi, 1991). The recognition and binding of CTL and target cell leads to a number of biochemical changes in the CTL resulting in the delivery of a "lethal hit" to the target cell (Podack and Kupfer, 1991). A number of mechanisms of CTL-mediated lysis have been proposed including CTL-induced apoptosis and the release of

cytotoxic granules containing perforin, granzymes, lymphotoxin and/or proteoglycans (reviewed in Podack and Kupfer, 1991).

Classically, T cells were divided into 2 major subclasses: $CD8^+$ CTL/T suppressor cells and $CD4^+$ T helper cells. However, it is now clear that the expression of the CD8 and CD4 molecules determines the type of MHC-encoded restricting element recognized by the TcR rather than the function of the T cell (Meuer et al., 1982). CD8 and CD4 are cell surface proteins that bind to conserved sequences of MHC class I and II, respectively (Brodsky and Guagliardi, 1991). Therefore, $CD8^+$ T cells recognize specific antigen in context of class I MHC molecules while $CD4^+$ T cells recognize antigen in context of class II MHC molecules.

In the majority of systems, anti-viral CTL induced upon infection are $CD8^+$. However, $CD4^+$ CTL have been detected in a number of viral systems including measles virus, influenza virus, VZV, CMV as well as HSV (reviewed in Schmid and Mawle, 1991).

B2. Characteristics of the human CTL response to HSV

B2(i). Phenotype of human anti-HSV CTL

Human HSV-specific CTL, originally described in 1980, were generated from HSV-seropositive donors by stimulating peripheral blood lymphocytes (PBL) with UV-irradiated HSV (UV-HSV) (Sethi et al., 1980). The presence of virus-specific, MHC class I-restricted CTL was demonstrated when bulk cultures of anti-HSV CTL lysed HSV-infected autologous skin fibroblasts (FB) but not uninfected or HSV-

infected HLA-A or B mismatched FB (Sethi et al., 1980). These results were confirmed using autologous EBV-transformed B lymphoblastoid cell lines (LCL) as target cells (Yasukawa et al., 1983). The presence of class II-restricted anti-HSV CTL was not tested. Later studies demonstrated that bulk cultures of anti-HSV CTL were comprised of class II-restricted $CD4^+CD8^-$ T cells only, capable of lysing HSV-infected LCL (Schmid, 1988), or a mixed population of $CD4^+CD8^-$ T cells and class I-restricted $CD4^-CD8^+$ T cells that lysed HSV-infected macrophages, monocytes (Torpey et al., 1989), keratinocytes (Cunningham and Noble, 1989) or LCL (Yasukawa et al., 1989). $CD8^+$ anti-HSV CTL were preferentially generated when PBL were stimulated with FB absorbed with UV-HSV while $CD4^+$ anti-HSV CTL were preferentially generated with UV-HSV as a stimulating agent (Yasukawa et al., 1989). This study demonstrated that $CD8^+$ HSV-specific CTL required cell-associated HSV for expansion in vitro while $CD4^+$ HSV-specific CTL required HSV antigen for expansion. Most recently, PBMC from HSV-seropositive donors were stimulated with autologous, PHA-activated, HSV-1 infected mononuclear cells. TcR-γ/δ $CD4^-CD8^-$ T cells were responsible for HSV-specific cytotoxic responses in some donors whereas TcR-α/β^+ $CD8^+$ T cells were primarily involved in other donors (Maccario et al., 1993). Although effector cells were virus-specific and lysis required the expression of HLA class I molecules on the surface of infected target cells, the response was HLA-unrestricted (Maccario et al., 1993). These studies suggested a role for both $CD8^+$ α/β^+ CTL and γ/δ^+ T cells in the human response to HSV. In summary, the phenotype of human HSV-specific CTL in bulk cultures

has depended on the mode of stimulation and/or the target cells used in cytotoxicity assays.

The phenotype of HSV-specific human CTL clones has also depended on the mode of stimulation of PBL. When PBL were subjected to repeated rounds of stimulation with UV-HSV-1, IL-2 and irradiated autologous PBL, HSV-specific CTL were exclusively $CD4^+CD8^-$ and class II-restricted (Yasukawa and Zarling, 1984a). Some clones were type-specific in that they would lyse HSV-1 infected LCL only, whereas others were type common, lysing both HSV-1 and HSV-2 infected LCL (Yasukawa and Zarling, 1984b). HSV-specific $CD8^+$ CTL clones were generated by repeated rounds of stimulation of peripheral blood mononuclear cells (PBMC) with irradiated, HSV-2 infected, PHA-activated PBMC plus allogeneic PBMC as feeder cells (Tigges et al., 1992). $CD8^+$ CTL clones were also type-common or type-specific (Tigges et al., 1992). Thus, as with bulk HSV-specific CTL, $CD8^+$ HSV-specific CTL clones were generated by stimulation of PBMC with cell-associated HSV while $CD4^+$ HSV-specific CTL clones were generated by stimulation with viral antigen.

HSV-specific CTL precursor frequencies have been determined by limiting dilution analysis. In one study, precursor frequencies of $CD4^+$ HSV-specific CTL ranged from 1 in 4000 to 1 in 8000 cells from peripheral blood stimulated with UV-HSV (Schmid, 1988). A second study determined the precursor frequencies of $CD4^+$ and $CD8^+$ CTL from a T cell-enriched population stimulated with UV-HSV to be approximately 1 in 5000 and 1 in 29,000 cells, respectively (Yasukawa et al.,

1989). However, when a T cell-enriched population was stimulated with UV-HSV-absorbed FB, precursor frequencies were approximately 1 in 20,000 for $CD4^+$ CTL and 1 in 10,000 $CD8^+$ CTL (Yasukawa et al., 1989). These results confirmed that the generation of $CD8^+$ and $CD4^+$ anti-HSV CTL is dependent on the mode of stimulation and that precursor frequencies of both T cell subsets are present in significant numbers in peripheral blood.

B2(ii). Specificity of human anti-HSV CTL

While extensive research has focussed on the antigen specificity of murine anti-HSV CTL, few studies address this issue in humans. To date, the research involving the specificity of human anti-HSV CTL has studied CTL clones generated by multiple rounds of stimulation of PBMC with UV-HSV or HSV-infected PBMC. Human $CD4^+$ CTL clones specific for HSV could be generated with exposure of PBL to UV-HSV or purified glycoproteins B (gB) or D (gD) of HSV-1 (Zarling et al., 1986a). Clones stimulated with UV-HSV or purfied gD also lysed autologous LCL infected with a recombinant vaccinia virus vector encoding gD (Zarling et al., 1986b). These results suggested that gB and gD can serve as target antigens for $CD4^+$ cloned CTL. Of seven $CD8^+$ CTL clones generated by stimulation of PBMC with HSV-infected PBMC from a single HSV-2 seropositive donor, one was specific for gD (Tigges et al., 1992). Further, by the use of drug blocking studies, one $CD8^+$ CTL clone recognized an immediate-early protein and five recognized internal virion proteins (Tigges et al., 1992). These results suggested that $CD8^+$ CTL clones are specific for diverse virion protein antigens.

B3. Changes in immune parameters during human HSV infections

Many laboratories have studied various cell-mediated immune functions during the acute and convalescent phases of HSV disease to correlate disease stage with a change in an immune parameter. One measure of cell-mediated immunity is proliferation of lymphocytes in response to antigen by ^{3}H-thymidine incorporation. While some studies have found that lymphoproliferation in response to HSV antigen is higher during acute disease relative to the disease-free interval (Kirchener et al., 1978; Tsutsumi et al., 1986), others have observed that proliferation is depressed during acute illness and rebounds after lesions have healed (El Araby et al., 1978; Vestey et al., 1990; Cauda et al., 1989). During acute illness, the depressed HSV-specific lymphoproliferative responses were abrogated by removal of $CD8^{+}$ T cells from PBMC (Vestey et al., 1990). Reconstitution of the $CD8^{+}$-depleted population with removed $CD8^{+}$ T cells suppressed lymphoproliferative responses (Vestey et al., 1990). An increase in the number of $CD8^{+}CD11^{+}$ T cells (suppressor cell phenotype) was noted during acute illness and corresponded to depressed lymphoproliferation in response to HSV antigen (Cauda et al., 1989). These results suggested that reduced lymphoproliferation to HSV during acute illness may be due to HSV-specific T suppressor cell function rather than lack of HSV-responsive lymphocytes (Vestey et al., 1990).

The alteration of HSV-specific CTL responses during disease progression has not been adequately studied. One study has reported a slight increase in

HSV-specific CTL activity during the acute phase of disease compared with convalescence, however, results were not statistically significant (Tsutsumi et al., 1986).

Unlike cell-mediated immune responses, no significant alterations in humoral immunity have been detected during the course of HSV disease. ELISA titres (Vestey et al., 1990) and the level of serum neutralizing antibodies specific for HSV (Douglas and Couch, 1970) did not change during acute illness as compared to convalescence.

Non-specific immune parameters have also been analysed. Lower NK cell activity during acute illness was observed (Kuo and Lin, 1990) which was directly related to a decrease in $CD16^+$ cell numbers (NK cell marker) (Cauda et al., 1989). IFN-α production was also suppressed when PBMC, isolated during acute illness, were stimulated with HSV antigen compared to PBMC isolated during convalescence (Kuo and Lin, 1990).

In summary, alterations in several immune parameters have been observed although it is not clear whether these changes are relevant in vivo or are merely markers of disease stages. Further, studies have focussed primarily on immune responses measured from peripheral blood which may not be indicative of responses occurring within a herpetic lesion.

C. MECHANISMS OF IMMUNE EVASION AND VIRUS-INDUCED IMMUNOSUPPRESSION

C1. Immune evasion by herpesviruses

The presence of free virus and virally-infected cells within a host renders the virus susceptible to recognition by the host's humoral and cellular immune systems. A number of viruses have developed specialized mechanisms of evasion of immune recognition and destruction allowing them to establish a persistent infection. As mentioned earlier, the hallmark of herpesviruses is their ability to establish a persistent infection by remaining latent in their hosts (Stevens, 1989). During latency, viral gene expression is restricted and no infectious virus is produced allowing the virus to go unnoticed by the immune system. HSV-1, HSV-2 and VZV remain latent with sensory neurons providing them with an enhanced avoidance from cell-mediated immunity since neurons are immunologically privileged due to the lack of MHC expression (Lampson, 1987).

Herpesviruses become sensitive to immune recognition upon reactivation when expression of viral genes and virus replication occurs. A common mechanism of viral evasion of immune surveillance is the down-regulation of MHC proteins. Down-regulation of MHC molecules on the surface of infected cells prevents their recognition and destruction by MHC-restricted virus-specific CTL. This phenomenon is well characterized in adenovirus-infected cells. The E3 region of adenovirus encodes a 19K protein that specifically binds to class I molecules preventing proper glycosylation and impeding their transport to the cell surface

(Gabthuler et al., 1990; Wold and Gooding, 1991). The E3 19K protein prevents the recognition and lysis of adenovirus-infected cells by anti-adenovirus CTL (Wold and Gooding, 1991).

Down-regulation of MHC protein expression has also been demonstrated in herpesvirus infections. Human CMV (HCMV) encodes a protein homologous to MHC class I that can associate with β_2-microglobulin (Browne et al., 1990; Beck and Barrell, 1988). A trimolecular complex containing an MHC class I protein, β_2-microglobulin and processed peptide is the structure recognized by the TcR (Brodsky and Guagliardi, 1991). Thus, by producing a protein that can associate with β_2-microglobulin, HCMV may prevent the formation of complexes and protect cells from CTL lysis, although this has not been demonstrated. HSV-2, and to a lesser extent, HSV-1, can also down-regulate the expression of MHC class I on the surface of infected cells (Jennings et al., 1985). The mechanism is not known but may be a consequence of virus-induced shut-off of host protein synthesis (Smibert and Smiley, 1990).

The complement system functions as an innate anti-viral defence mechanism (alternative complement system) or as an effector arm of the adaptive immune system (classical complement system). Complement activation leads to the lysis of virally-infected cells or destruction of free virus. HSV encodes proteins that can inhibit alternative and classical complement activation. A complex of HSV glycoproteins E (gE) and I (gI), that is expressed on the surface of infected cells, acts as an Fc receptor binding to the Fc portion of IgG (Johnson et al., 1988).

HSV-FcR is thought to participate in antibody bipolar bridging reducing the effectiveness of antibody-dependent cellular cytotoxicity (ADCC) (Dubin et al., 1991) or by preventing complement-mediated lysis or Fc-facilitated phagocytosis (Bell et al., 1990). HSV glycoprotein C (gC) acts as a C3b receptor and can bind to complement component C3b (Harris et al., 1990). Binding of gC to C3b inhibits the amplification of the classical or alternative complement pathway and subsequent inhibition of complement-mediated lysis of free virus or infected cells (Harris et al., 1990). Vaccinia virus also encodes a protein, VCP, that binds to complement component C4b inhibiting the classical, but not alternative, complement system (Kotwal et al., 1990). Therefore, inhibition of complement activation may be a common mechanism viruses use to evade destruction allowing them to persist in their hosts.

C2. Herpesvirus-induced immunosuppression

Another means assisting viruses in spreading and establishing persistent infections in their hosts is by directly suppressing the host's immune system. Herpesviruses are not only remarkable in immune evasion but also in suppressing important immune responses that would normally be involved in controlling and clearing the viral infection (Rinaldo, 1990).

Herpesvirus-induced immunosuppression is normally associated with CMV and EBV infections. Direct infection of lymphocytes and monocytes with HCMV may result in immune impairments observed during HCMV infection, although this has not been proven in vivo (Banks and Rouse, 1992). Other viruses directly

infect lymphoid cells leading to suppression of function or elimination. HIV infects $CD4^+$ T cells leading to their elimination, although the mechanism is not known. Measles virus is also lymphotropic and suppresses T cell function (McChesney et al., 1988). Human herpesvirus 6 (HHV-6), another member of the herpesvirus family, can directly infect NK cells leading to their destruction (Lusso et al., 1993). Therefore, the direct infection of cells by viruses is a common mechanism of suppressing responses of the innate and adaptive immune systems.

The mechanism of immunosuppression by EBV is also unresolved. One potential mechanism of EBV-induced immunosuppression may be mediated by an EBV protein, BCRF1, that shares homology with IL-10, a cytokine known to inhibit cytokine synthesis by T helper-1 (TH-1) cells (Del Prete et al., 1993). BCRF1 has IL-10 activity including the inhibition of IFN-gamma production from T cells and NK cells (Hsu et al., 1990). Suppressing the function of cells involved in inflammatory immune responses may be an important mechanism permitting primary EBV infection and establishment of latency.

Evidence for the immunosuppressive effects of HSV infection is slowly emerging. HSV-induced immunosuppressive effects have only been observed in non-specific immune functions. HSV-infection of monocytes suppressed their ability to present antigen to T cells (Hayward et al., 1993). Further, IL-1α and TNF-β secretion from HSV-infected macrophages was depressed in response to phorbol-ionomycin stimulation compared to uninfected macrophages (Hayward et al., 1993). These results demonstrate that HSV can interfere with monocyte

accessory functions essential for T cell stimulation.

NK cell function is also inhibited by HSV-infected cells; the incubation of NK cells with HSV-infected fibroblasts or endothelial cells profoundly inhibited the lysis of NK-sensitive target cells (Confer et al., 1990). Inhibition of NK cell function was dependent on cell-to-cell contact and appeared to require the synthesis of viral glycoproteins (Confer et al., 1990). The inhibition of NK cell and monocyte functions may be an important means by which HSV can avoid destruction by first-line defence mechanisms.

RATIONALE AND PURPOSE OF STUDY

Recombinant vaccinia viruses encoding individual HSV glycoproteins have been used to study the specificity of human and murine anti-HSV CTL. Recombinant adenovirus vectors encoding glycoproteins have been used successfully to study the specificity of murine anti-HSV CTL and protection from lethal HSV challenge. The present study utilized recombinant adenovirus vectors encoding HSV glycoproteins B (gB) or D (gD) to determine their usefulness in studying the specificity of human anti-HSV CTL using autologous EBV-transformed B lymphoblastoid cell lines (LCL) as target cells. The source of anti-HSV CTL was bulk cultures of peripheral blood mononuclear cells (PBMC) from HSV-seropositive donors stimulated with a single in vitro exposure to HSV-1.

The results of the first part of this study demonstrated that recombinant adenovirus vectors expressed low levels of insert gene products in infected LCL thereby preventing their lysis by gB- and gD-specific CTL. Thus, an alternate cell line was required to study the specificity of anti-HSV CTL employing recombinant adenoviruses. Autologous fibroblasts were chosen as target cells because they were permissive to adenovirus infection and easily obtained from the donors. Surprisingly, HSV-infected fibroblasts (HSV-FB) were not lysed by anti-HSV CTL, and further, the exposure of anti-HSV CTL to HSV-FB rendered the CTL unable to lyse normally sensitive target cells. The focus of this study was subsequently directed at analysing the mechanism of inhibition of CTL lytic function by exposure to HSV-FB. The inhibition of CTL-mediated lysis by HSV-FB may be an important means of virus-induced immunosuppression allowing the virus to spread and persist in immunocompetent individuals.

CHAPTER 2

Analysis of Human Anti-Herpes Simplex Virus Cytotoxic T Lymphocytes Specific for Glycoproteins B and D in Polyclonal Cultures

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ABSTRACT

To date, glycoproteins B (gB) and D (gD) of herpes simplex virus (HSV) are the only HSV proteins that have been shown to act as target antigens for human anti-HSV cytotoxic T lymphocytes (CTL). However, these gB- and gD-specific CTL were T cell clones generated by repeated stimulation of peripheral blood mononuclear cells (PBMC) with HSV antigens (1) or HSV-infected cells (2). The presence of gB- and gD-specific CTL in polyclonal cultures of anti-HSV-1 CTL has not been examined. Therefore, we determined whether PBMC stimulated with a single in vitro exposure to HSV-1 would lyse autologous B-lymphoblastoid cell lines (LCL) infected with vaccinia virus recombinants encoding gB (vgB11) or gD (vgD52) of HSV-1. Of six HSV-seropositive donors tested, only one donor generated $CD4^+$ and $CD8^+$ CTL which lysed autologous LCL infected with HSV-1, vgB11 and vgD52. HSV-1-stimulated PBMC from five donors lysed HSV-1-infected LCL only. Thus, although gB and gD can serve as target antigens for bulk cultures of human anti-HSV-1 CTL, the majority of the bulk CTL response in most patients is directed at HSV antigens other than gB and gD.

Herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) can remain latent in neuronal tissue after primary infection, and when activated by one of a variety of stimuli, can cause oral or genital lesions. T cell-mediated immunity appears to play an important role in resistance to HSV infections in both mice and humans. In mice, adoptive transfer of HSV-immune T cells provided resistance to HSV infection (3). Severe HSV infections can occur in humans with suppressed T cell immunity (4,5), suggesting a role for T cells in controlling primary infection and reactivation.

Anti-HSV CTL clones established by stimulating PBMC from HSV-seropositive donors with UV-HSV were exclusively HLA class II-restricted $CD4^+CD8^-$ T cells (6), while clones generated by stimulation of PBMC with HSV-infected PHA-activated PBMC were solely $CD8^+$ and HLA class I-restricted (2). The stimulation of PBMC with UV-HSV for 5 days generated HLA-DR restricted, $CD4^+CD8^-$ anti-HSV CTL capable of lysing HSV-infected LCL (7). However, it has

also been shown that stimulation of PBMC with UV-HSV generates both subsets of T cells with the ability to lyse autologous HSV-infected monocytes and macrophages (8), HSV-infected PHA-stimulated PBMC (9), or HSV-infected keratinocytes (10). A study by Yasukawa and colleagues (11) has shown that although $CD4^+CD8^-$ CTL are preferentially generated by UV-HSV, $CD4^-CD8^+$ CTL are also present and can be preferentially generated by stimulation of PBMC with UV-HSV-absorbed fibroblasts. The generation of either subset of T cell in bulk cultures or T cell clones has depended on the mode of stimulation of PBMC; $CD4^+$ CTL have predominated when PBMC are stimulated with HSV antigen while $CD8^+$ CTL predominate when cell-associated virus is used as the stimulating agent (1,2,11).

While many studies have focussed on the phenotype of human HSV-specific CTL, few studies address their specificity. Work by Zarling and colleagues (1) has demonstrated that HSV-1 glycoprotein B (gB) and D (gD) stimulated PBMC from HSV-seropositive individuals in vitro as measured by IL-2 production and lymphoproliferation. Class II-restricted $CD4^+CD8^-$ CTL clones produced by stimulation with purified gB or gD were able to lyse autologous HSV-infected LCL (1). These studies indicated that memory CTL which recognized gB and gD were present in the peripheral blood of the HSV-seropositive individuals tested. Of seven $CD8^+$ HSV-specific CTL clones isolated from a single individual with frequently recurrent genital herpes, one was specific for gD, five were specific for an internal virion protein and one was specific for a nonvirion protein (2). gD-

specific $CD8^+$ CTL could also be demonstrated in an HSV-2 seropositive individual from bulk PBMC stimulated with irradiated HSV-infected PHA-stimulated PBMC (2).

In the present study, we measured gB- and gD-specific CTL responses in polyclonal cultures of HSV-specific CTL from six HSV-seropositive donors. Polyclonal CTL cultures were generated by stimulating PBMC with 0.1 PFU/cell HSV-1 strain F for 7 days (12). Target cells used were autologous EBV-transformed B lymphoblastoid cell lines (LCL) that were uninfected or infected overnight with 10 PFU/cell HSV-1, or vaccinia viruses encoding gB (vgB11) (13) or gD (vgD52) (14) of HSV-1 (viruses kindly provided by B. Moss, NIH). Polyclonal cultures were tested for lytic activity in standard ^{51}Cr-release assays as previously described (12).

Of the six HSV-seropositive donors tested, only one (KL) had measureable gB- and gD-specific CTL activity (Table 1). In the remaining five donors, lysis of vgB11- and vgD52-infected LCL was not different than the lysis of VVNP-infected LCL (control vaccinia virus recombinant encoding the nucleoprotein of Pichinde virus [15]). Lysis was virus specific and MHC-restricted since uninfected LCL and HSV-infected allogeneic LCL were not lysed (Table 1 and data not shown). These data indicate that although gB and gD can act as target antigens for human anti-HSV-1 CTL, the majority of the bulk CTL response in most patients was directed at HSV antigens other than gB and gD.

The phenotype of gB- and gD-specific CTL from donor KL was determined

by depleting either T cell subset from the bulk cultures. CD16- and CD56-positive cells (NK cells) were depleted using antibody and complement and the CD4- or CD8-positive cells were removed using antibody and magnetic beads as previously described (12). NK-depleted cultures killed LCL infected with HSV-1, vgB11 and vgD52 but not LCL infected with VVNP demonstrating that these polyclonal CTL contained gB- and gD-specific CTL (Table 2). Depletion of either the CD4- or the CD8-positive population reduced the killing of LCL infected with HSV-1, vgB11 and vgD52 (Table 2). Cultures depleted of $CD4^+$ or $CD8^+$ T cells contained less than 1% $CD4^+$ cells and less than 5% $CD8^+$ cells, respectively, as determined by FACS analysis (data not shown). These results indicate that CTL-mediated lysis of HSV-1- infected LCL and gB- and gD-expressing LCL was mediated by both CD4- and CD8-positive T cells.

Human anti-HSV CTL have been generated using UV-HSV, UV-HSV-absorbed fibroblasts or HSV-infected PHA-stimulated PBMC as the stimulating agent. In this study, we used live HSV to stimulate bulk human PBMC and demonstrated that $CD4^+$ and $CD8^+$ HSV-specific CTL were generated. Live virus was used at a low MOI and was not cytopathic for bulk human PBMC. Replication of HSV is apparently not cytopathic to T cells, B cells or macrophages isolated from fresh blood unless the cells are stimulated with mitogens or antigens (16).

Our results demonstrate that HSV gB and gD can act as target antigens for polyclonal human anti-HSV-1 CTL generated from PBMC and stimulated with one exposure to HSV-1 for 7 days. Further, both $CD4^+$ and $CD8^+$ CTL could mediate

lysis of gB- and gD-expressing LCL. However, as this gB- and gD-specific CTL response could be detected in only 1 of 6 HSV-seropositive donors tested, it appears that other viral components are involved in recognition of HSV by human anti-HSV-1 CTL. This recognition of individual HSV proteins is paralleled in the human anti-influenza CTL response where 1 of 5 donors tested had detectable nucleoprotein-specific CTL (17). Polyclonal cultures of human anti-EBV CTL also displayed broad antigen specificities depending on HLA type, although EBV latent proteins 3A, 3B and 3C were recognized most frequently (18).

gB and gD were originally tested as potential CTL targets in humans because they could serve as CTL target antigens in inbred mouse strains (13,14,19). Further, viral proteins expressed on the surface of infected cells were originally studied as potential CTL targets because it was believed that the TcR recognized membrane-bound viral protein associated with MHC. However, it is now clear that the TcR recognizes peptide fragments of viral proteins bound to MHC molecules on the cell surface. Thus, peptides generated from viral proteins expressed intracellularly or in the membrane can act as CTL antigens. In the murine HSV model, the immediate-early protein ICP27, found intracellularly, is a target antigen for H-2^d mice (20). Tigges and colleagues (2) have shown that internal virion proteins can act as target antigens for human HSV-specific $CD8^+$ CTL clones. Thus, it is evident that the study of the specificity of human HSV-specific CTL should not be restricted to HSV glycoproteins.

This is the first demonstration of gB- and gD-specific human CTL generated

from a single in vitro exposure to live HSV-1. Detection of gB- and gD-specific CTL in other individuals may require CTL cloning to detect low frequencies of these CTL. While gB and gD can act as target antigens for human anti-HSV CTL, other major target antigens have yet to be identified and are likely to be characterized with the utilization of recombinant viral vectors.

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TABLE 1. Recognition of LCL Infected with vgB11 and vgD52 by PBMC Stimulated with HSV-1

PBMC from HSV-seropositive donors were stimulated for 7 days with HSV-1 prior to use in a ^{51}Cr-release assay. Target cells were ^{51}Cr-labeled autologous uninfected (un) LCL or LCL infected overnight with HSV-1, VVNP, vgB11 or vgD52. Results represent percent specific ^{51}Cr-release at an effector:target ratio of 60:1 for GS and CP, 50:1 for KL and MW, and 30:1 for BL and TH. Underlined values are significantly different that the lysis of LCL infected with VVNP ($p<0.05$; student's t test).

DONOR	Target Cell				
	un	HSV	VVNP	vgB11	vgD52
KL	1	34	5	<u>33</u>	<u>35</u>
GS	2	41	2	1	6
BL	5	29	11	12	11
TH	5	19	0	5	6
MW	0	15	4	2	0
CP	5	21	2	0	4

TABLE 2. Determination of the Phenotype of gB- and gD-specific Human CTL

Polyclonal anti-HSV-1 CTL from KL were depleted of NK cells (CD16- and CD56-positive cells) and CD4- or CD8-positive cells just prior to use in a ^{51}Cr-release assay. Results represent percent specific ^{51}Cr release at an effector:target ratio of 60:1 for NK-depleted effectors; effectors were not readjusted after depletion of CD4- or CD8-positive cells.

Infected LCL Target	Cell population depleted		
	CD16/CD56	CD16/CD56 CD4	CD16/CD56 CD8
HSV-1	41	17	27
VVNP	7	3	4
vgB11	27	12	16
vgD52	19	10	9

CHAPTER 3

RESTRICTED INFECTION OF HUMAN B LYMPHOBLASTOID CELLS BY RECOMBINANT ADENOVIRUSES: INABILITY TO SERVE AS CYTOTOXIC T LYMPHOCYTE TARGETS

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Running head: Restricted infection of human LCL by adenoviruses

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SUMMARY

Recombinant adenovirus vectors have been used successfully to study the specificity of murine anti-herpes simplex virus (HSV) cytotoxic T lymphocytes (CTL). In light of this, we tested the ability of recombinant adenovirus-infected EBV-transformed B-lymphoblastoid cell lines (LCL) to serve as targets for human HSV-specific CTL *in vitro*. Anti-HSV CTL specifically lysed LCL infected with HSV and recombinant vaccinia viruses encoding HSV glycoproteins B (gB) (vgB11) and D (gD) (vgD52) but not LCL infected with recombinant adenoviruses encoding gB (AdgB) or gD (AdgD). Immunoprecipitation of AdgB- and AdgD-infected LCL revealed that gB and gD were expressed. However, glycoprotein expression was detected at later times post-infection and required a higher MOI than LCL infected with vgB11 or vgD52. FACS analysis revealed that less than 2% of AdgB- or AdgD-infected LCL expressed the relevant glycoprotein. To confirm the low expression of the inserted gene product in LCL infected with recombinant adenoviruses, LCL were infected with a recombinant adenovirus encoding the β-galactosidase gene, LacZ (Ad5LacZ). Although β-galactosidase activity was detected in Ad5LacZ-infected LCL, levels were significantly lower than in permissive cells. Further, only 0.01% of cells stained positively for β-galactosidase activity. Therefore, the restricted ability of recombinant adenoviruses to infect and express in human LCL accounts for their inability to serve as targets for CTL *in vitro*.

INTRODUCTION

Anti-viral CTL are involved in controlling many viral infections including those caused by members of the herpesvirus family. While many studies have focussed on the phenotype of human HSV-specific CTL (Schmid, 1988; Torpey *et al.*, 1989; Yasukawa *et al.*, 1989), few studies address their specificity. Work by Zarling *et al.* (1986*a*) has demonstrated that HSV-1 glycoprotein B (gB) and D (gD) stimulated peripheral blood lymphocytes (PBL) from HSV-seropositive individuals *in vitro* as measured by IL-2 production and ^{3}H-thymidine uptake. Class II-restricted $CD4^{+}CD8^{-}$ CTL clones produced by stimulation with purified gB or gD were able to lyse autologous HSV-infected LCL (Zarling *et al.*, 1986*a*). These studies indicated that memory CTL which recognized gB and gD were present in the peripheral blood of HSV-seropositive individuals. LCL infected with a recombinant vaccinia virus expressing gD were recognized by anti-HSV CTL clones produced by stimulation with HSV-1 (Zarling *et al.*, 1986*b*). gD-specific $CD8^{+}$ CTL could be demonstrated in an HSV-2 seropositive individual from bulk peripheral blood mononuclear cells (PBMC) stimulated with irradiated HSV-infected PHA-stimulated PBMC (Tigges *et al.*, 1992). Further, seven $CD8^{+}$ HSV-specific CTL clones were isolated from a single individual with frequently recurrent genital herpes: one was specific for gD, five were specific for an internal virion protein and one was specific for a nonvirion protein (Tigges *et al.*, 1992). We have shown that in one of six HSV-seropositive individuals tested, polyclonal cultures of HSV-specific CTL contained gB- and gD-specific CTL which lysed LCL infected with

recombinant vaccinia viruses encoding gB (vgB11) and gD (vgD52) (Posavad and Rosenthal, unpublished results). gB- and gD-specific CTL were composed of $CD4^+$ and $CD8^+$ T cells.

The specificity of anti-viral CTL has largely been studied using recombinant viral vectors such as vaccinia virus. In mice, recombinant adenovirus vectors encoding individual HSV glycoproteins have aided in elucidating target antigens. HSV-specific CTL generated in $H\text{-}2^b$ or $H\text{-}2^d$ mouse strains efficiently lysed syngeneic cell lines infected with a recombinant adenovirus encoding gB (AdgB) (Witmer *et al.*, 1990). Conversely, spleen cells isolated from mice immunized with AdgB lysed HSV-infected cells (Hanke *et al.*, 1991). Recombinant adenovirus vectors were also used to map a region in gB which contains an immunodominant epitope recognized by $H\text{-}2^b$ anti-HSV CTL (Hanke *et al.*, 1991). Recently, mice immunized intranasally with AdgB generated protective immunity from subsequent mucosal challenge with HSV-2 (Gallichan *et al.*, 1993).

With the success of recombinant adenoviruses in mice, it was anticipated that these vectors would assist in characterizing the specificity of human HSV-specific CTL. In the present study, HSV-specific CTL were generated from HSV-seropositive donors and tested for lytic activity against autologous LCL infected with adenoviruses encoding gB (AdgB) or gD (AdgD) of HSV-1. LCL were chosen as the target cells since it was reported that LCL support adenovirus replication (Lavery *et al.*, 1987; Horvath *et al.*, 1991). Our results demonstrate that recombinant adenoviruses do not readily infect and express in human LCL, and

thus, alternate susceptible cells must be used as CTL targets.

MATERIALS AND METHODS

Cell lines and viruses. LCL were established and maintained as previously described (Posavad & Rosenthal, 1992). HeLa cells and human foreskin fibroblasts (HFF) were maintained in α-MEM containing 10% FBS.

HSV-1 strain F (obtained from P. Spear, University of Chicago) was propagated and titred on Vero cell monolayers maintained in α-MEM containing 8% FBS. VgB11 and vgD52, recombinant vaccinia viruses encoding genes for gB and gD, respectively (Cantin *et al.*, 1987; Cremer *et al.*, 1985) (obtained from B. Moss, NIH) and VVNP (recombinant vaccinia virus encoding nucleoprotein [NP] gene from Pichinde virus) (Ozols *et al.*, 1990) were propagated and titred on CV-1 maintained in Dulbecco's modified essential medium containing 8% FBS. AdgB-2 (hereafter named AdgB) (Johnson *et al.*, 1988), AdgD (Zheng B, Graham FL, Johnson DC, Hanke T, McDermott MR, and Prevec L, unpublished results) and Ad5LacZ (kindly provided by L. Prevec, McMaster Universitiy) (Prevec L, unpublished results), recombinant adenovirus vectors containing genes in the E3 region encoding HSV-1 gB, gD and β-galactosidase, respectively, and AddlE3 (Ad5 with deletion in the E3 region) (Haj-Ahmad & Graham, 1986) (kindly provided by F. Graham, McMaster University) were propagated and titred on 293 or HeLa.

Generation of anti-HSV-1 CTL. Anti-HSV-1 CTL were generated from HSV seropositive donors as previously described (Posavad & Rosenthal, 1992). Lytic

activity of anti-HSV-1 CTL was determined by standard ^{51}Cr-release assays as previously described (Posavad & Rosenthal, 1992); target cells were ^{51}Cr-labeled LCL infected with viruses at various times post-infection.

^{35}S-Methionine labeling, immunoprecipitation and SDS-PAGE. LCL were left uninfected or were infected for various times with HSV-1 or a vaccinia virus recombinant at an MOI of 10 or with a recombinant adenovirus vector at an MOI of 50. During the last 2 hours of infection, the cells were washed twice in medium 199 lacking methionine (199-meth) and metabolically labeled with 50 μCi ^{35}S-methionine (ICN Biomedicals Canada Ltd., St. Laurent, Quebec) in 1 ml 199-meth. Cells were then washed twice in PBS and cell lysates prepared on ice with 1 ml RIPA buffer, pH 7.2 (50 mM Tris, 0.15M NaCl, 1% Triton X-100, 1% Na deoxycholate, 0.1% SDS, 100 KIU/ml aprotonin [Sigma Chemical Co., St. Louis, MO]). HSV glycoproteins were immunoprecipitated from the cell extracts with protein A-Sepharose (Pharmacia Chemicals, Dorval, Quebec) and either 15βB2 (monoclonal antibody specific for gB) (Johnson *et al.*, 1988) or a rabbit polyclonal serum specific for gD (Eisenberg *et al.*, 1982) for 4 hours at 4°C. Immunoprecipitates were washed three times in cold RIPA buffer, 50 μl of SDS sample buffer was added, and samples were heated at 100°C for 5 min. Samples (40 μl) were loaded onto a continuous 10% polyacrylamide gel and electrophoresed at 70V overnight. Gels were then fixed in water-methanol-acetic acid (50:50:7) for 1 hour, infused with ENLIGHTNING (New England Nuclear Corp., Boston, MA) for 15 min, dried and exposed to Kodak XAR film.

FACS analysis. LCL were mock infected or were infected overnight with HSV-1, VVNP, vgB11 or vgD52 at an MOI of 10 or with AddIE3, AdgB or AdgD at an MOI of 50. Cells were pelleted and incubated with a 1/1000 dilution of rabbit HSV-specific antiserum or rabbit gB-specific antiserum (Eberle & Courtney, 1980) on ice for 30 minutes. Cells were washed twice with PBS containing 0.1% FBS (PBS-FBS), pelleted and incubated with a 1/250 dilution of FITC-conjugated goat anti-rabbit IgG (secondary antibody) on ice for 30 minutes. Cells were washed twice with PBS-FBS and fixed with 1% paraformaldehyde. Some cells were left unstained, incubated with secondary antibody only or with normal rabbit serum (NRS) and secondary antibody. Cells (10,000) were analysed on a Becton Dickinson FACScan. Gates were set using NRS control for each sample so approximately 2% of cells were positive.

Use of Ad5LacZ to study infectivity of LCL by adenoviruses. Ad5LacZ is a recombinant adenovirus vector containing the LacZ gene which encodes β-galactosidase (L. Prevec, unpublished results). Two methods were used to detect the expression of β-galactosidase in LCL. Firstly, a colorimetric enzyme assay was used to detect the breakdown of 0-nitrophenyl-B-D-galactopyranoside (ONPG) by β-galactosidase present in infected-cell lysates (Miller, 1972). LCL, HeLa cells and human foreskin fibroblasts (HFF) were left uninfected or were infected for various times with Ad5LacZ. Cells were pelleted and resuspended in 300 μl of a solution contaning 250 mM Tris HCL (pH 7.8), 1 mM PMSF and 0.5% NP40. Samples were frozen at -70°C until assayed. Samples were vortexed at 4°C for

20 minutes and centrifuged at 12,000 rpm for 10 minutes. Supernatant (40 μl) was added to 350 μl of a solution containing 10 mM KCl, 1 mM Na phosphate (pH 7.5) and 50 mM 2-mercaptoethanol for 5 minutes at 37°C followed by the addition of 132 μl ONGP (4 g/l in 100 mM Na phosphate, pH 7.5) for 1 hour. The reaction was terminated with 172 μl of 1 M Na_2CO_3. The presence of β-galactosidase produces a yellow color which can be detected by spectrophotometry at 420 nm.

In the second method, Ad5LacZ-infected cells were examined for the presence of β-galactosidase in situ using the chromogenic substrate X-gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside) which is converted to a blue precipitate in the presence of β-galactosidase. LCL and HeLa cells were infected with Ad5LacZ as stated above. Following infection, cytospins were prepared onto microscope slides using 100 μl of HeLa cells (5 x 10^5/ml) or LCL (1 x 10^6/ml). Slides were incubated for 6 hours at 37°C with 100 μl of X-gal solution (73.5 mM X-gal, 0.2 M NaPi, 1 mM $MgCl_2$, 5 mM potassium ferricyande, 5 mM potassium ferrocyanide, 0.5 μM SDS). Slides were viewed under a light microscope to determine the percentage of blue staining cells.

RESULTS

Expression of HSV proteins in recombinant virus-infected LCL. We tested the ability of LCL infected with recombinant adenovirus vectors to express the relevant HSV glycoprotein. LCL were infected with a recombinant adenovirus or vaccinia virus vector for various times and expression of HSV glycoproteins was detected

by immunoprecipitating ^{35}S-methionine-labeled glycoproteins from cellular lysates with HSV-specific antibodies. The expression of HSV-specific glycoproteins was undetectable in LCL infected with a recombinant adenovirus vector at an MOI of 10 (data not shown), and therefore, LCL were infected at an MOI of 50. AdgB-infected LCL expressed gB at 24 and 36 hours post-infection, in contrast to HSV-1-infected LCL which expressed gB at 6 hours post-infection (Figure 1A). Expression of gB from AdgB-infected cells could be detected up to 72 hours post-infection (data not shown). Expression of gB from vgB11-infected cells was detectable at 12 hours post-infection and levels increased by 24 hours (Figure 1B).

The expression of gD from AdgD-infected LCL was detected 24 and 48 hours post-infection and was still apparent up to 72 hours post-infection (Figure 2A), while the expression of gD from vgD52-infected cells could be detected at 18 hours post-infection (Figure 2B). Thus, each of the vectors expressed the appropriate HSV glycoprotein in LCL. However, HSV glycoprotein expression was detected earlier in LCL infected with a recombinant vaccinia virus at an MOI of 10 as compared with LCL infected with an adenovirus vector at an MOI of 50.

Susceptibility of recombinant adenovirus-infected LCL to human anti-HSV-1 CTL. Figure 3 shows that targets infected for 24 hours with HSV-1, vgB11 or vgD52 were lysed by HSV-specific CTL from an HSV-seropositive individual. In contrast, LCL infected for 24 or 48 hours with AdgB or AdgD were not lysed (Figure 3). Mock infected LCL and LCL infected with control viruses, VVNP and AddlE3, were not lysed (Figure 3), nor were HSV-1-infected allogeneic LCL (data

not shown). Thus, anti-HSV-1 CTL were capable of lysing LCL expressing gB and gD infected with recombinant vaccinia viruses but were unable to lyse LCL expressing gB or gD from recombinant adenoviruses.

FACS analysis of LCL infected with recombinant viruses. To determine the percentage of AdgB- and AdgD-infected LCL that expressed gB and gD on their surface, infected LCL were stained with HSV-specific antiserum and FITC-conjugated goat anti-rabbit IgG. FACS analysis revealed that while LCL infected with HSV-1 or vgD52 stained positively for HSV-specific proteins, LCL infected with vgB11, AdgB or AdgD did not stain positively above normal rabbit serum staining (Table 1). Although LCL infected with vgB11 did not stain positively with the rabbit HSV-specific antiserum, gB expression could be detected with rabbit gB-specific antiserum (Table 1). In contrast, gB-specific antiserum did not detect gB on the surface of AdgB-infected LCL (Table 1). When a larger number of cells were analyzed (50,000) in order to detect a small population of cells expressing gB, staining of AdgB-infected LCL was no different than the staining of AddlE3-infected LCL (data not shown). Similarly, no positive staining was detected in LCL infected with AdgB and AdgD for 48 hours (data not shown). Thus, the cell surface expression of gB and gD from AdgB- and AdgD-infected LCL was significantly reduced relative to LCL infected with HSV-1, vgB11 and vgD52.

Utilization of Ad5LacZ to study expression of insert gene in recombinant adenovirus-infected LCL. Although adenoviruses have been previously shown to infect and express adenovirus proteins in infected LCL (Lavery *et al.*, 1987), in

our hands, LCL expressed low levels of protein encoded by insert genes. To further study the expression of genes inserted into the E3 region of adenovirus type 5 (Ad5) in LCL, we utilized an Ad5LacZ vector. The Ad5LacZ vector contains the LacZ gene, encoding β-galactosidase, in the E3 region of Ad5. Figure 4 demonstrates that HeLa cells or human foreskin fibroblasts (HFF) infected with 5 PFU/cell or 10 PFU/cell Ad5LacZ, respectively, expressed high levels of β-galactosidase activity at 24, 48 or 72 hours post-infection. In contrast, LCL infected with 50 PFU/cell Ad5LacZ contained low, but detectable, levels of β-galactosidase activity at 24 hours post-infection that increased marginally by 48 and 72 hours post-infection (Fig. 4).

In order to enumerate LCL expressing β-galactosidase following infection with Ad5LacZ, cells were stained with X-gal in situ. Cytospins of HeLa cells infected with 5 PFU/cell Ad5LacZ for 48 hours demonstrated greater than 95% of cells staining positively for β-galactosidase activity. In contrast, only 0.01% of LCL infected for 48 hours with 50 PFU/cell Ad5LacZ stained positively for β-galactosidase activity. These results demonstrate that only a small percentage of Ad5LacZ-infected LCL expressed the inserted gene product. Thus, human LCL do not appear to be permissive for infection with recombinant adenovirus vectors and the low level of expression of the inserted gene product in infected LCL may make them poor CTL targets.

DISCUSSION

In this study, we analysed the lysis of LCL infected with recombinant adenovirus vectors expressing individual HSV glycoprotein genes by human HSV-specific CTL. Although recombinant adenovirus vectors expressed detectable levels of the relevant HSV glycoprotein in LCL by immunoprecipitation, these cells were not recognized by anti-HSV-1 CTL. gB- and gD-specific CTL were clearly present in polyclonal cultures of anti-HSV-1 CTL because LCL infected with vgB11 and vgD52 were lysed. gB and gD expression could not be detected in LCL infected with AdgB or AdgD by FACS analysis suggesting that the expression of gB and gD expression occurred in a small percentage (<2%) of LCL. Low expression of recombinant adenovirus insert genes in LCL was confirmed using Ad5LacZ where 0.01% of cells were positive for β-galactosidase activity. These results suggest that LCL infected with recombinant adenoviruses were not lysed by anti-HSV CTL because too few cells were infected and expressed HSV glycoproteins.

LCL were chosen as the cell type in our system for several reasons. Firstly, LCL are easily generated from the blood of most individuals and are an accessible source of autologous and heterologous human target cells. Secondly, studies have demonstrated the presence of group C (including Ad5) adenovirus DNA sequences in LCL (Horvath *et al.*, 1986) and that established human B cell lines, including LCL, support adenovirus type 5 replication (Lavery *et al.*, 1987; Horvath *et al.*, 1991). Thirdly, the adenovirus constructs used here have the HSV genes

inserted into the E3 region of Ad5 and are thus unable to produce the E19 protein. The E3 E19 protein is the protein responsible for binding to human class I antigens and impeding their transport to the cell surface (Gabathuler *et al.*, 1990; Paabo *et al.*, 1986) However, even in the presence of the E3 E19 protein, Ad5 infection has been shown not to cause major decreases in surface class I antigen expression until late in infection when cell death was imminent (Routes & Cook, 1990). Finally, human LCL express both class I and class II antigens and thus, CD4- and CD8-mediated lysis could be detected.

The expression of HSV gB and gD in LCL infected with adenovirus or vaccinia virus vectors was examined after immunoprecipitation and SDS-PAGE. AdgB- and AdgD-infected LCL expressed detectable levels of HSV glycoprotein by 24 hours post-infection and continued to express at 48 and 72 hours post-infection using an MOI of 50. HSV-1 and recombinant vaccinia viruses infected LCL expressed detectable levels of gB and gD by 6 and 18 hours post-infection, respectively, using an MOI of 10. Therefore, the adenovirus recombinants expressed HSV glycoproteins at later times and higher amounts of virus were required in order to detect protein expression.

LCL infected with recombinant adenovirus vectors were not recognized by anti-HSV-1 CTL at any time point tested. The inability of polyclonal anti-HSV CTL to lyse LCL infected with recombinant adenoviruses appears to be due to the restricted infection and expression in human LCL. FACS analysis revealed that levels of gB and gD in AdgB- and AdgD-infected LCL were not detectable above

background levels and further, the expression of β-galactosidase from Ad5LacZ-infected LCL confirmed that only 0.01% of cells expressed the inserted gene product.

Lavery *et al.* (1987) showed that LCL were permissive for infection by adenoviruses. Ad5 infection of T and B cell lines supported viral DNA replication, RNA synthesis, cell surface expression of viral protein and assembly of Ad5 virions. Although Ad5 replication was characteristic of each cell type, up to 60% of cells were infected (Lavery *et al.*, 1987). The results of the present study indicate that LCL are poor targets for recombinant Ad5 infection and expression of genes inserted into the E3 region of Ad5. The infection of LCL by recombinant adenoviruses reflects the infection of freshly isolated PBMC by adenoviruses. Human PBMC were shown to be non-permissive for adenovirus infection, although a subpopulation was infectable (Horvath & Weber, 1988). A similar situation may exist with human LCL; a certain phenotype of LCL in the population, perhaps depending on activation state or stage in the cell cycle, may permit adenovirus replication. However, the expression of adenovirus insert genes, normally expressed in permissive cell lines, is restricted in the majority of LCL.

We are currently investigating human skin fibroblasts infected with the recombinant adenovirus vectors as potential targets for anti-HSV-1 CTL. Human foreskin fibroblasts infected with Ad5LacZ expressed levels of β-galactosidase activity comparable to Ad5LacZ-infected HeLa cells. Further, fibroblasts infected with AdgB or AdgD expressed abundant levels of the relevant HSV glycoproteins

as detected by immunoprecipitation and immunofluorescence (Posavad & Rosenthal, unpublished results). However, a positive control for this system is not available since HSV-infected fibroblasts are resistant to lysis by human anti-HSV-1 CTL (Posavad & Rosenthal, 1992). HSV-infected fibroblasts are not only resistant to lysis by anti-HSV-1 CTL but can inhibit the lytic activity of both anti-HSV-1 CTL and alloantigen-specific CTL by a contact-dependent mechanism (Posavad & Rosenthal, 1992).

Although our studies indicate that recombinant adenoviruses have limited utilization to study specificity of human CTL when LCL are used as target cells, there is great potential for recombinant adenoviruses encoding viral genes to be used as vaccines. Oral vaccination of military recruits with adenovirus has been successful against upper respiratory tract infections with no adverse reactions (Meiklejohn, 1983). Mice immunized intraperitoneally with AdgB2 were protected from lethal challenge with HSV (McDermott *et al.*, 1989). Recently, mice immunized intranasally with AdgB developed mucosal and systemic immunity which protected them from subsequent challenge with HSV-2 (Gallichan *et al.*, 1993). These findings suggest that recombinant adenoviruses may be useful as human vaccines.

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Table 1. Expression of HSV-specific proteins in LCL infected with recombinant adenovirus and vaccinia virus vectors[a]

Virus	MOI	% of cells positive[b]		
		NRS	anti-HSV	anti-gB
mock	-	<2	<2	6
HSV-1F	10	<2	38	26
VVNP	10	<2	<2	6
vgB11	10	<2	<2	34
vgD52	10	<2	17	ND[c]
AddlE3	50	<2	<2	4
AdgB	50	<2	<2	5
AdgD	50	<2	<2	ND

[a]LCL were mock infected or were infected for 24 hours with 10 PFU/cell HSV-1F or vaccinia virus recombinant or with 50 PFU/cell of adenovirus recombinant. Cells were stained with normal rabbit serum (NRS), rabbit HSV-specific antiserum (anti-HSV) or with rabbit gB-specific antiserum (anti-gB) followed by FITC-conjugated goat anti-rabbit IgG. [b]A gate was set so cells stained with NRS were approximately 2% positive.

[c]Not done.

FIG. 1. Time course of expression of gB in AdgB and vgB11 infected LCL. LCL were left uninfected (un) or were infected for 6 hours with 10 PFU HSV-1, (A) infected with 50 PFU AdgB for 12, 24 or 36 hours, or (B) infected with 10 PFU vgB11 for 6, 12 or 24 hours. HSV-gB was immunoprecipitated from cell lysates with protein A-Sepharose and 15βB2. The immature or precursor form of gB is indicated by pgB.

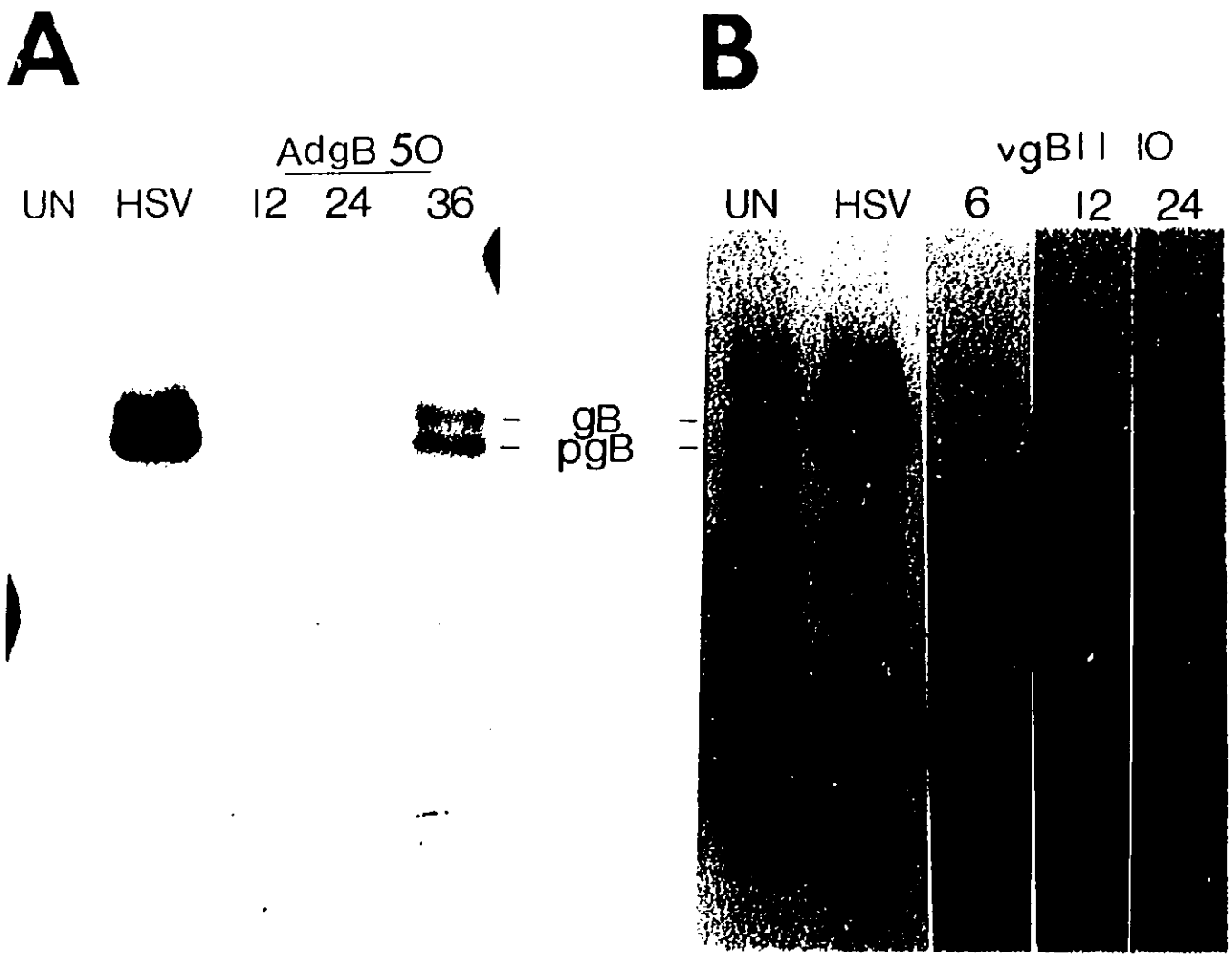
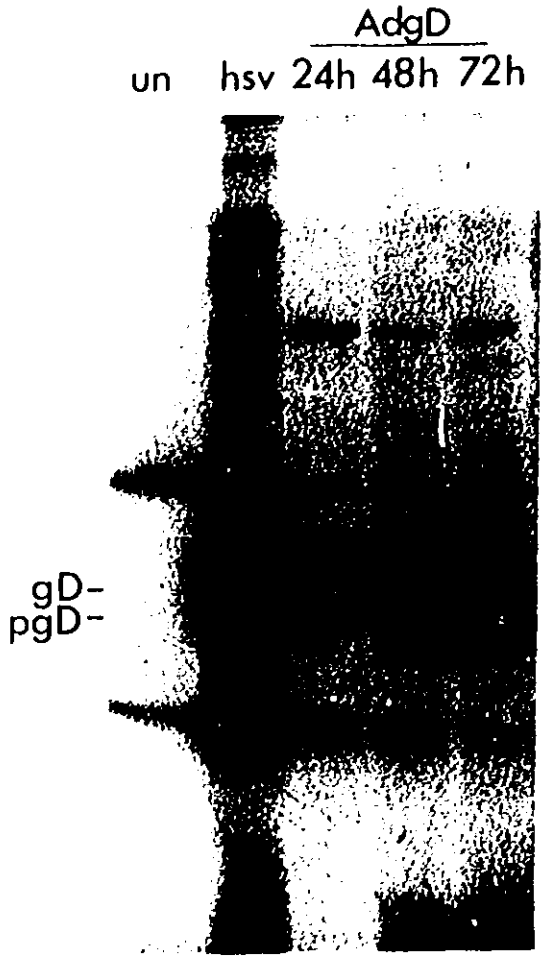

A
AdgB 50
UN
HSV
12
24
36
gB
pgB
B
vgB11 10
UN
HSV
6
12
24

FIG. 2. Time course of expression of gD in AdgD and vgD52 infected LCL. LCL were left uninfected (un), infected for 6 hours with 10 PFU HSV-1, (A) infected with 50 PFU AdgD for 24, 48 or 72 hours, or (B) infected with 10 PFU vgB11 (vgB) or vgD52 (vgD) for 6 or 18 hours. HSV-gD was immunoprecipitated with a polyclonal serum specific for gD. The immature or precursor form of gD is indicated by pgD.

A


AdgD
un
hsv
24h
48h
72h
gD-
pgD-

B

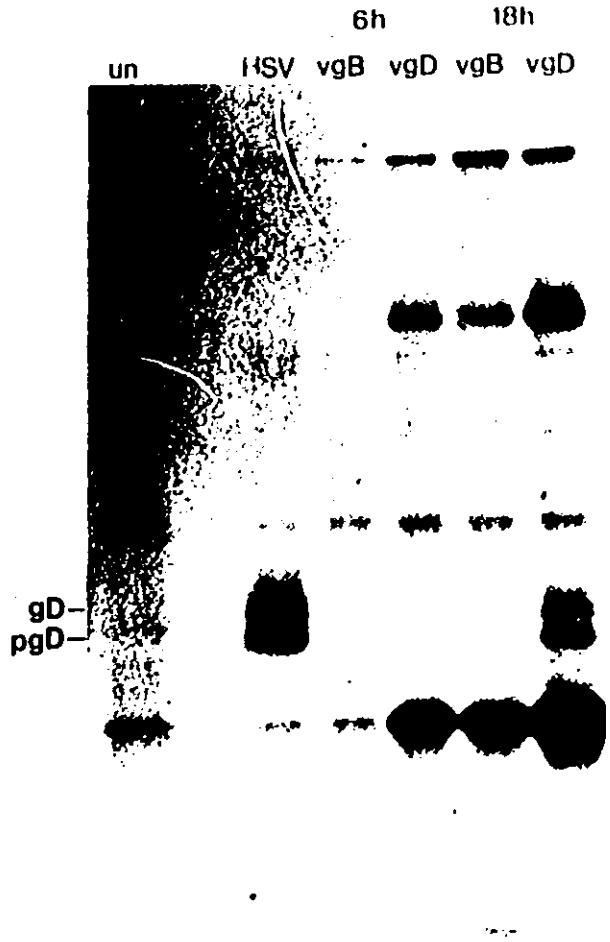

6h
18h
un
HSV
vgB
vgD
vgB
vgD
gD-
pgD-

FIG. 3. Human anti-HSV-1 CTL lyse LCL infected with vgB11 and vgD52 but not LCL infected with AdgB or AdgD. PBMC from KL stimulated in vitro with HSV-1 were tested for cytotoxic activity against LCL which were mock infected or infected for 24 hours with 10 PFU HSV-1, VVNP, vgB11 or vgD52 or infected for 24 or 48 hours with 50 PFU AddIE3, AdgB or AdgD. Effector:target ratio was 60:1; error bars represent the standard deviation from the mean from triplicate wells.

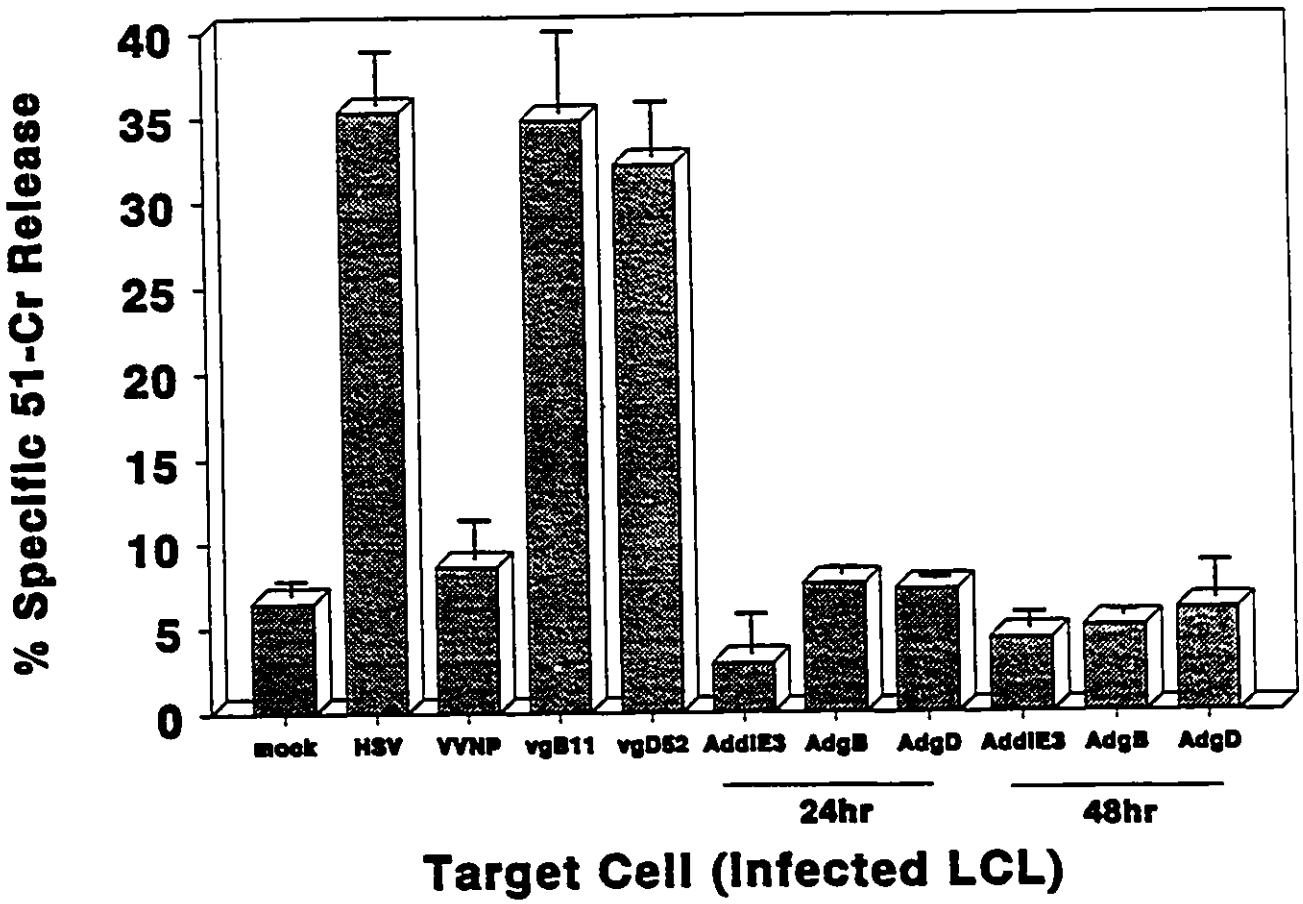
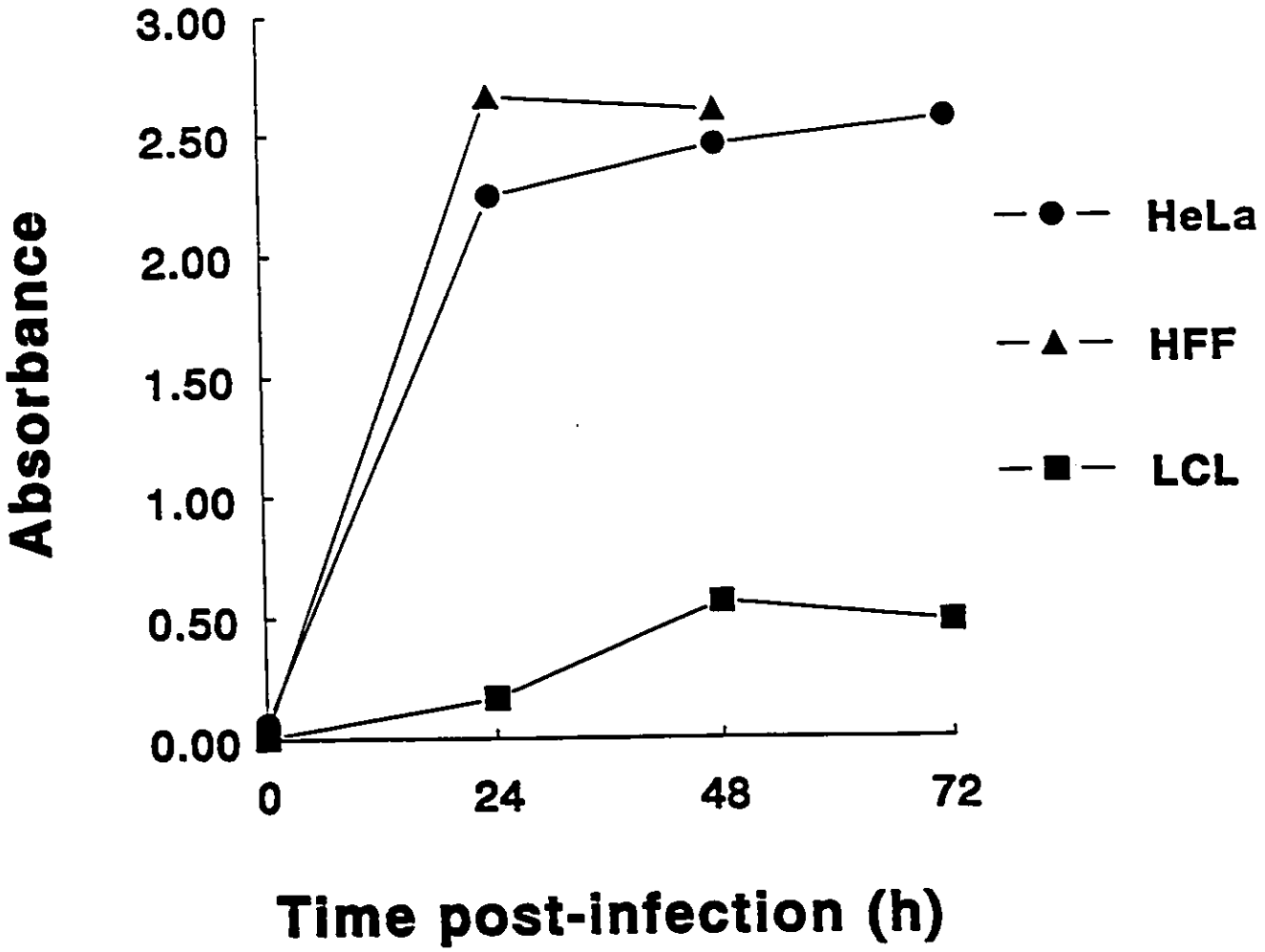

% Specific 51-Cr Release
40
35
30
25
20
15
10
5
0
mock
HSV
VVNP
vgB11
vgD52
AddIE3
AdgB
AdgD
AddIE3
AdgB
AdgD
24hr
48hr
Target Cell (Infected LCL)

FIG. 4. Expression of β-galactosidase in AdLacZ-infected cell lines. HeLa cells, HFF and LCL were left uninfected (0 hours) or were infected for 24, 48 or 72 hours with AdLacZ (5 PFU/cell for HeLa cells; 50 PFU/cell for LCL). Cell lysates were tested for the presence of β-galactosidase activity by conversion of ONPG as outlined in Materials and Methods. The absorbance was read at 420 nm.


3.00
2.50
2.00
1.50
1.00
0.50
0.00
Absorbance
0
24
48
72
Time post-infection (h)
HeLa
HFF
LCL

CHAPTER 4

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Herpes Simplex Virus-Infected Human Fibroblasts Are Resistant to and Inhibit Cytotoxic T-Lymphocyte Activity

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We examined the ability of human anti-herpes simplex virus (HSV) cytotoxic T lymphocytes (CTL) to lyse autologous human fibroblasts infected with HSV. In contrast to HSV-infected human Epstein-Barr virus-transformed B cells (LCL), which were lysed by HLA-restricted anti-HSV CTL, autologous fibroblasts infected with HSV were resistant to lysis. This resistance was not due to a lack of infectivity or production of HSV proteins since greater than 90% of the cells were infected and expressed abundant levels of viral proteins. HSV-infected human fibroblasts were also tested for susceptibility to lysis by alloantigen-specific CTL. Although allogeneic LCL and uninfected allogeneic fibroblasts were killed, human fibroblasts infected with HSV demonstrated a time-dependent resistance to lysis by alloantigen-specific CTL. HSV-infected human fibroblasts were not resistant to all forms of cell-mediated cytotoxicity since they were sensitive to antibody-dependent cellular cytotoxicity. Although one may suspect that the resistance of HSV-infected human fibroblasts to anti-HSV CTL and alloantigen-specific CTL-mediated lysis was due to a lack of major histocompatibility complex expression, Confer et al. (Proc. Natl. Acad. Sci. USA 87:3609–3613, 1990) previously demonstrated that incubation of human natural killer and lymphokine-activated killer cells with monolayers of human fibroblasts infected with HSV "disarmed" the killers in that they were unable to lyse sensitive target cells. We extend their results and show that incubation of anti-HSV CTL or alloantigen-specific CTL with uninfected fibroblasts did not affect their lytic activity, whereas CTL incubated with HSV-infected fibroblasts for 2 to 6 h rendered the CTL incapable of lysing their normally sensitive target cells. Indeed, human fibroblasts infected for merely 2 h with HSV were able to profoundly inhibit the cytotoxic activity of alloantigen-specific CTL. Thus, HSV-infected human fibroblasts are not inherently resistant to lysis by anti-HSV CTL or alloantigen-specific CTL, but rather contact of CTL with HSV-infected fibroblasts resulted in inactivation of the CTL. The inactivation of CTL appears to be HSV specific since incubation of alloantigen-specific CTL in sandwich assays with fibroblasts infected with HSV type 1 (HSV-1) or HSV-2 resulted in inactivation, whereas incubation of CTL with fibroblasts infected with adenovirus or vaccinia virus had no effect. Further, although incubation of alloantigen-specific CTL in sandwich assays with HSV-infected fibroblasts resulted in inhibition of CTL activity, exposure of CTL in Transwell cultures to cell-free supernatant from HSV-infected fibroblasts did not mediate this inhibitory effect. Thus, the inhibition of CTL by HSV-infected fibroblasts is not mediated by a soluble factor but rather appears to require cell-to-cell contact. Gaining an understanding of the mechanism by which HSV-infected human fibroblasts inhibit CTL activity may be relevant to our understanding of virus-induced immunosuppression and how viruses escape from immune surveillance and provide insight into inactivation and anergy of mature cytotoxic effector cells.

A number of viruses are known to establish persistent infections in immunocompetent individuals. To achieve this, a virus must persist within cells in the host, and these virus-infected cells must evade immune surveillance and destruction, especially from antiviral cytotoxic T lymphocytes (CTL). To avoid detection and destruction by antiviral CTL, viruses have evolved numerous mechanisms. One example of this is the downregulation of major histocompatibility complex (MHC) expression that occurs in cells infected with certain adenovirus subtypes (29). More recently, it was shown that viruses may also escape immune surveillance by mutating crucial T-cell epitopes recognized by CTL. This was demonstrated for lymphocytic choriomeningitis virus both in vivo (17) and in vitro (1) and for human immunodeficiency virus (15). Alternatively, viruses may be immunosuppressive by directly or indirectly interfering with cells of the immune system (12). Human immunodeficiency virus, for example, infects CD4$^+$ T cells, which results in their depletion and subsequent immunosuppression in infected individuals.

Cell-mediated immunity is known to play an important role in controlling the spread and severity of herpes simplex virus (HSV) infection in humans. Indeed, the severity of infection with HSV is inversely correlated with the competency of the host cellular immune response (6). The role of T cells in controlling HSV infections is further implicated by the serious, sometimes life-threatening, primary or recurrent infections that can develop in immunocompromised individuals, particularly transplant recipients treated with immunosuppressive drugs, such as cyclosporin A (14, 16, 21), and individuals with AIDS (20, 26).

Despite measurable cell-mediated immune responses to HSV in immunocompetent individuals, the virus can establish a latent infection in neuronal tissue, and frequent recurrences can still occur. Understanding the nature of this HSV-host interaction is made more difficult by the fact that herpesviruses themselves are immunosuppressive (2, 22).

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Indeed, it is not clear whether immunosuppression leads to reactivation of HSV or whether HSV reactivation results in immunosuppression.

In this study, we analyzed the ability of human skin fibroblasts (FB) infected with HSV to be recognized by HSV-specific CTL. FB infected with HSV (HSV-FB) were previously reported to be lysed by $CD8^+$ anti-HSV CTL generated after in vitro exposure of peripheral blood lymphocytes to irradiated FB absorbed with UV-inactivated HSV (30). FB have also been shown to serve as target cells for CTL specific for cytomegalovirus, another member of the herpesvirus family (3, 4). It was thus surprising to find that, in contrast to HSV-infected human Epstein-Barr virus-transformed B-cell lines (LCL), which were readily lysed by autologous anti-HSV CTL, HSV-FB were resistant to lysis by these CTL. We further showed that HSV-FB were resistant to alloantigen-specific CTL-mediated lysis. Although the resistance of HSV-FB to anti-HSV CTL and alloantigen-specific CTL-mediated lysis might be interpreted as resulting from a lack of appropriate MHC-viral epitope expression by the target cells, our findings were reminiscent of those reported by Confer et al. (5). These investigators used sandwich assays to demonstrate that incubation of human natural killer (NK) and lymphokine-activated killer (LAK) cells with a monolayer of HSV-FB or HSV-infected human endothelial cells, but not uninfected FB or endothelial cells, "disarmed" the killers in that they were unable to lyse sensitive target cells.

Here we support and extend the findings of Confer et al. (5) and demonstrate that HSV-FB inactivate human anti-HSV CTL and alloantigen-specific CTL by a contact-dependent mechanism resulting in the inability of CTL to lyse normally sensitive targets. Thus, the resistance of HSV-FB to anti-HSV CTL and alloantigen-specific CTL is not due to an inherent resistance of the target cells; rather, the resistance results from the ability of HSV-FB to inhibit a wide variety of cytotoxic effector-cell activities. The ability of human HSV-FB to inhibit local CTL-mediated lysis may be a mechanism of virus-induced immunosuppression that permits HSV to spread and persist in immunocompetent hosts after primary infection or reactivation of latent HSV.

MATERIALS AND METHODS

Cell lines and viruses. LCL were established by infecting 2.5×10^6 peripheral blood mononuclear cells (PBMC) overnight with 2.5 ml of cell-free supernatant of B95-8 cells. Cells were then pelleted and resuspended in 5 ml of RPMI 1640 medium containing 20% heat-inactivated fetal bovine serum (FBS), 102 U of penicillin G per ml, 10 μg of streptomycin sulfate per ml, 2 mM L-glutamine, 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid) (all from GIBCO Laboratories, Grand Island, N.Y.), and 1 μg of cyclosporin A (Sandoz Canada, Montreal, Quebec, Canada) per ml in a 25-cm^2 flask. After 7 days, cells were pelleted, resuspended, and maintained in RPMI 1640 medium containing 10% FBS, 102 U of penicillin G per ml, 10 μg of streptomycin sulfate per ml, 2 mM L-glutamine, and 10 mM HEPES (hereafter termed culture medium). Human skin FB were obtained by skin biopsy from the underside of the forearm and were maintained in α minimal essential medium containing 10% FBS, 102 U of penicillin G per ml, 10 μg of streptomycin sulfate per ml, 2 mM L-glutamine, and 10 mM HEPES (hereafter termed FB medium). K562 cells, an NK cell-sensitive human erythroleukemia cell line, were maintained in culture medium.

HSV type 1 (HSV-1) strain F (obtained from P. Spear, University of Chicago) and HSV-2 strain 333 were propagated, and titers were determined on Vero cell monolayers maintained in α minimal essential medium containing 8% FBS. Vaccinia virus strain WR (obtained from the American Type Culture Collection) was grown and titers were determined on CV-1 cells. Human adenovirus type 5 (obtained from F. Graham, McMaster University) was grown in HeLa cells, and titers were determined on 293 cells.

[^{35}S]methionine labeling, immunoprecipitation, and SDS-polyacrylamide gel electrophoresis. FB were left uninfected or were infected for various times with 10 PFU of HSV per cell. In the last 2 h of infection, the cells were washed twice in medium 199 lacking methionine and metabolically labeled with 50 μCi of [^{35}S]methionine (ICN Biomedicals Canada Ltd., St. Laurent, Quebec, Canada) in 1 ml of medium 199 lacking methionine. Cells were then washed twice in phosphate-buffered saline (PBS) and cell lysates were prepared on ice with 1 ml of radioimmunoprecipitation assay (RIPA) buffer (pH 7.2) (50 mM Tris, 0.15 M NaCl, 1% Triton X-100, 1% Na deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 100 KIU of aprotonin [Sigma Chemical Co., St. Louis, Mo.] per ml). HSV-specific proteins were immunoprecipitated from cell extracts with protein A-Sepharose (Pharmacia Chemicals, Dorval, Quebec, Canada) and rabbit anti-HSV-1 immune serum (kindly provided by W. Rawls, McMaster University) for 4 h at 4°C. Immunoprecipitates were washed three times in cold RIPA buffer, 50 μl of SDS sample buffer was added, and samples were heated at 100°C for 5 min. Samples (40 μl) were loaded onto a continuous 10% polyacrylamide gel and electrophoresed at 70 V overnight. Gels were fixed in water-methanol-acetic acid (50:50:7) for 1 h, infused with Enlightning (New England Nuclear Corp., Boston, Mass.) for 15 min., dried, and exposed to Kodak XAR film.

Immunofluorescence. Subconfluent monolayers of FB grown on coverslips were left uninfected or were infected for various times with 10 PFU of HSV per cell. Cells on coverslips were fixed in cold acetone for 10 min and allowed to air dry before the coverslips were mounted onto microscope slides. Slides were washed twice with PBS containing 0.1% bovine serum albumin (PBS-BSA), and 40 μl of a 1:8 dilution of rabbit anti-HSV-1 immune serum was added. Slides were incubated at 37°C for 30 min and then washed six times with PBS-BSA. Slides were then incubated for 30 min at 37°C with 40 μl of a 1:20 dilution of fluorescein isothiocyanate-goat anti-rabbit immunoglobulin G. Slides were washed six times with PBS-BSA, 1 drop of glycerol-based aqueous mounting solution was added, and slides were covered with a coverslip. Fluorescence was viewed with a Leitz inverted fluorescence microscope.

Generation of effector cells. Anti-HSV CTL were prepared as described previously (31) with modifications. Briefly, PBMC were isolated from HSV-seropositive donors by using Ficoll-Paque (Pharmacia) and resuspended at 10^6/ml in culture medium containing 0.02 mM 2-mercaptoethanol (Sigma Chemical Co., St. Louis, Mo.). Ten million cells were cultured at 37°C in 5% CO_2 in 25-cm^2 upright flasks with 10^6 PFU of HSV. After 7 days, cells were harvested as primary effectors.

Alloantigen-specific CTL were generated as above by replacing HSV with 10^6 irradiated (2,000 rads) allogeneic PBMC as stimulators.

Cell-mediated cytotoxicity assays. Standard chromium release assays were used to assess CTL-mediated cytotoxicity. LCL and FB, used as target cells, were left uninfected or

were infected for various times with 10 PFU of HSV per cell. In the final 1.5 h of infection, cells were labeled with 200 μCi of $Na_2{}^{51}CrO_4$ (New England Nuclear Corp.), washed, and counted. Target cells (1×10^4 to 2×10^4) were added to effectors at various effector/target cell ratios in 96-well flat-bottomed microtiter plates (Nunclon, Roskilde, Denmark) and incubated for 5 h at 37°C in 5% CO_2. Target cells were also incubated in culture medium alone (spontaneous release) or with 1 N HCl (maximal release). After 5 h of incubation, 100 μl of supernatant was removed from each well and its radioactivity was counted in a gamma counter. The percent specific ^{51}Cr release was calculated as follows: [(cpm experimental release − cpm spontaneous release)/(cpm maximal release − cpm spontaneous release)] × 100, where cpm is counts per minute. The spontaneous release was always less than 30% of the maximal release. In some cases, OKT3, a monoclonal antibody specific for CD3 which blocks killing by $CD3^+$ cells, was added to wells before the addition of target cells.

In some experiments, cytotoxicity was determined by a sandwich assay as described by Confer et al. (5) with slight modifications. Briefly, uninfected FB and FB infected for 2, 4 or 20 h with 10 PFU of HSV were trypsinized, and 10^4 cells per well were added to 96-well flat-bottomed plates. FB were allowed to adhere and recover for 1 to 2 h. Afterward, effectors were added to the FB for 2, 4, or 6 h before the addition of ^{51}Cr-labeled target cells in a ^{51}Cr release assay.

The ability of effector cells to lyse HSV-FB via antibody-dependent cellular cytotoxicity (ADCC) was also determined. Briefly, human PBMC were isolated and tested for cytotoxicity against ^{51}Cr-labeled autologous uninfected FB or HSV-FB in the absence or presence of rabbit anti-HSV immune serum (1:80 dilution), which was added at the start of a ^{51}Cr release assay.

Depletion of T-cell and NK-cell subsets. For elimination of $CD4^+$, $CD8^+$, $CD56^+$, or $CD16^+$ cells, effectors were resuspended at 3×10^6/ml in PBS-BSA containing anti-leu3a (anti-CD4), anti-leu2b (anti-CD8), anti-leu19 (anti-CD56), or anti-leu11b (anti-CD16) (Becton Dickinson Immunocytometry Systems, Mountain View, Calif.) at 1:100 and incubated for 30 min at 4°C. Cells were washed three times with PBS-BSA and resuspended at 5×10^6/ml. DYNABEADS M-450 coated with goat anti-mouse immunoglobulin G (DYNAL Inc., Great Neck, N.Y.) were added to cells treated with anti-leu3a, anti-leu2b, and anti-leu19 at a bead/cell ratio of 10:1. The cells and beads were incubated for 1 h at 4°C with continuous rocking. The beads with attached cells were then magnetically removed, and the remaining cells were resuspended in culture medium and used as effectors. Two milliliters of a 1:10 dilution of Low-Tox-H rabbit complement (Cedarlane, Hornby, Ontario, Canada) was added to cells treated with anti-leu11b and incubated for 1 h at 37°C. Cells were then washed three times with PBS-BSA, resuspended in culture medium, and used as effectors.

Role of soluble FB factors in CTL inhibition. To determine whether soluble factors were involved in inhibition of CTL activity, we obtained conditioned supernatant from uninfected FB or from FB infected overnight with 10 PFU of HSV and passed through a 0.22-μm-pore-size filter. Conditioned supernatant (250 μl) was then added to effectors for 4 h before the addition of ^{51}Cr-labeled targets. Additionally, Transwells (Costar, Cambridge, Mass.) were used to maximize exposure of effector cells to FB supernatants without direct contact. FB (5×10^4 per well) were grown in a 24-well cluster plate (Costar). Cells were left uninfected or were infected with 10 PFU of HSV per cell in 0.5 ml of α-minimal essential medium with 5% FBS. After 30 min of absorption of HSV, the inoculum was removed and 1 ml of FB media was added per well. A 6.5-mm-diameter tissue culture-treated Transwell with a 0.4-μm pore size (Costar) was placed in each well, and 200 μl of FB medium was added to the Transwell. Culture supernatant was allowed to equilibrate overnight through the porous polycarbonate filter. Alloantigen-specific CTL (200 μl) were added to the Transwell (1×10^6 to 2×10^6 effectors per Transwell) for 4 h; this was followed by their removal and addition to ^{51}Cr-labeled target cells.

TABLE 1. Resistance of HSV-FB to anti-HSV CTL-mediated lysis

Target[a]	Cell population depleted[b]		
	$CD16^+$ + $CD56^+$	$CD16^+$ + $CD56^+$ + $CD4^+$	$CD16^+$ + $CD56^+$ + $CD8^+$
auto LCL	8	8	3
auto HSV-LCL	38	16	20
allo HSV-LCL	7	6	3
auto FB	4	3	1
auto HSV-FB	6	2	3

[a] Target cells were uninfected autologous (auto) LCL or FB, or auto LCL, auto FB, or allogeneic (allo) LCL infected for 6 h with 10 PFU of HSV per cell.

[b] PBMC from an HSV-seropositive donor were stimulated with HSV for 7 days and were depleted of NK cells ($CD16^+$ and $CD56^+$ cells) and $CD4^+$ or $CD8^+$ cells just before use in a ^{51}Cr release assay. Percent specific ^{51}Cr release is shown at an effector:target cell ratio of 50:1; effector cells were not readjusted after $CD4^+$ or $CD8^+$ cells were depleted.

Statistical analysis. Results were analyzed by a Student's two-tailed *t* test with MINITAB statistical software. A *P* value of <0.05 was taken to be significant.

RESULTS

Resistance of HSV-FB to lysis by human anti-HSV CTL. HSV-FB were tested for sensitivity to lysis by human anti-HSV CTL. Human anti-HSV CTL, generated from PBMC of HSV-seropositive donors and depleted of $CD16^+$ and $CD56^+$ NK cells, were tested for lytic activity against autologous LCL and HSV-FB. Although anti-HSV CTL clearly lysed LCL infected for 6 h with HSV, no lysis of HSV-FB was detected (Table 1). Anti-HSV CTL were virus specific and MHC restricted since neither uninfected autologous LCL nor HSV-infected allogeneic LCL were lysed (Table 1). Depletion of either $CD4^+$ or $CD8^+$ cells from the bulk population of effectors reduced, but did not eliminate, the killing of HSV-infected autologous LCL (Table 1). These results demonstrate that both $CD4^+$ and $CD8^+$ anti-HSV CTL were present in the effector population. Although Table 1 displays the lytic activity of anti-HSV CTL from a single HSV-seropositive donor, anti-HSV CTL generated from all donors tested did not lyse autologous HSV-FB (data not shown). Further, human FB infected for 18 and 24 h with HSV were also fully resistant to lysis by autologous anti-HSV CTL (data not shown). Thus, our results demonstrate that human FB infected with HSV are resistant to lysis by autologous anti-HSV CTL which are capable of lysing virus-infected LCL. Our results do not support those of Yasukawa et al. (30), who showed that human FB infected overnight with HSV were sensitive to $CD8^+$ human anti-HSV CTL.

Expression of HSV glycoproteins in HSV-FB. To ensure

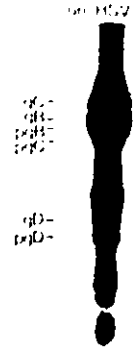

FIG. 1. Immunoprecipitation of HSV glycoproteins in HSV-FB. Human FB were left uninfected (un) or were infected for 20 h with 10 PFU of HSV per cell (HSV). HSV proteins were immunoprecipitated with rabbit anti-HSV-1 immune serum and protein A-Sepharose. The immature forms of glycoproteins B (gB), C (gC), and D (gD) are indicated by pgB, pgC, and pgD, respectively.

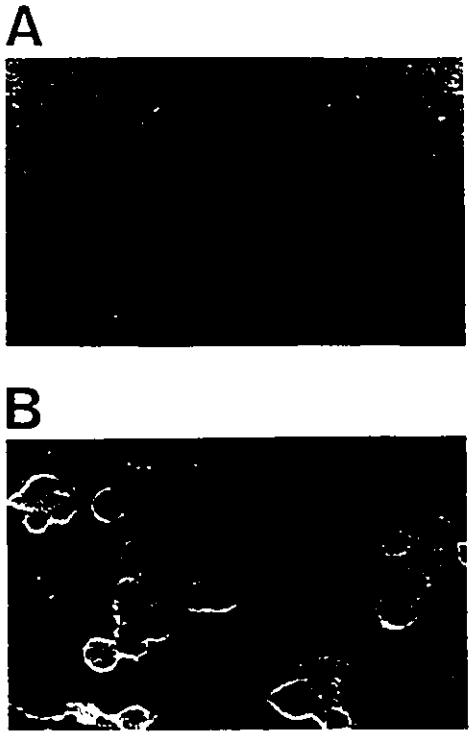

FIG. 2. Expression of HSV proteins in human FB. Human FB grown on coverslips were left uninfected (A) or were infected for 20 h with 10 PFU of HSV per cell (B) and fixed in cold acetone. Coverslips were incubated with rabbit anti-HSV-1 immune serum and then incubated with fluorescein isothiocyanate-labeled goat anti-rabbit immunoglobulin G. Cells incubated with fluorescein isothiocyanate-labeled goat anti-rabbit immunoglobulin G alone displayed no detectable fluorescence.

that the resistance of HSV-FB to $CD8^+$ anti-HSV CTL was not due to lack of infectability or nonproduction of HSV proteins, we determined the expression of HSV glycoproteins in HSV-FB. Figure 1 demonstrates that FB infected with HSV express abundant levels of HSV-specific glycoproteins. To determine the proportion of FB infectible with HSV, we performed immunofluorescence on monolayers of human FB infected with HSV. Greater than 90% of FB stained positively for HSV-specific proteins, whereas no positively staining cells were observed in a monolayer of uninfected FB (Fig. 2). These results demonstrate that HSV infects and expresses abundant levels of viral proteins in human FB and thus that resistance of HSV-FB to anti-HSV CTL was not due to a lack of infectivity or viral protein production.

Sensitivity of HSV-infected human FB to ADCC. Although HSV-FB were not lysed by human anti-HSV CTL, HSV-FB infected for 6 h with HSV were sensitive to ADCC (Fig. 3). In contrast to the NK cell-sensitive K562 cell line, uninfected FB and HSV-FB were weakly sensitive to lysis by fresh PBMC. Addition of rabbit anti-HSV immune serum to the FB and PBMC resulted in a marked increase in lysis of HSV-FB but not uninfected FB (Fig. 3). These results demonstrate that infection of FB with HSV renders them sensitive to the lytic machinery triggered through the Fc receptor on human NK cells and that HSV-FB are not resistant to all forms of cell-mediated lysis.

Resistance of HSV-FB to alloantigen-specific CTL. Human alloantigen-specific CTL, depleted of $CD16^+$ and $CD56^+$ cells, lysed allogeneic LCL and uninfected FB (Fig. 4). However, the lysis by alloantigen-specific CTL of FB infected for 4 h with HSV was reduced relative to the lysis of uninfected FB, and infection for 20 h rendered the cells fully resistant to lysis (Fig. 4). Thus, human FB infected with HSV demonstrated a time-dependent resistance to lysis by alloantigen-specific CTL. Alloantigen-specific CTL were specific for allogeneic cells since neither autologous LCL nor FB were killed (Fig. 4). Further, depletion of $CD16^+$ and $CD56^+$ NK cells was successful as demonstrated by the low level of lysis of K562 cells (Fig. 4). Therefore, although sensitive to ADCC, human FB infected with HSV for over 4 h were fully resistant to both autologous human anti-HSV CTL and alloantigen-specific CTL.

Inactivation of CTL by HSV-FB. Although one may interpret the resistance of HSV-FB to anti-HSV CTL- and alloantigen-specific CTL-mediated lysis as a result of lack of MHC expression, Confer et al. (5) demonstrated that incubation of human NK and LAK cells with a monolayer of human FB or endothelial cells infected with HSV, but not uninfected FB or endothelial cells, disarmed the killers in that they were unable to lyse sensitive target cells. To determine whether a similar phenomenon was occurring with human CTL, we conducted sandwich assays. For these assays, anti-HSV CTL were incubated for 2 h with human FB that had previously been infected with HSV for 20 h or left uninfected. Afterwards, chromium-labeled target cells were added and cytotoxicity was measured. Results shown in Fig. 5 demonstrate that anti-HSV CTL incubated on uninfected FB clearly lysed HSV-infected autologous LCL. In contrast, incubation of anti-HSV CTL on HSV-FB rendered these CTL incapable of lysing HSV-infected LCL (Fig. 5). The inhibition of anti-HSV CTL lytic activity by incubation with HSV-FB ranged from 61% at an effector/target cell ratio of 50:1 to 81% at an effector/target cell ratio of 25:1 relative to the lysis mediated by anti-HSV CTL incubated on uninfected FB ($P < 0.05$).

Human alloantigen-specific CTL were also sensitive to the inhibitory effects of HSV-FB. Incubation of alloantigen-specific CTL with HSV-FB inhibited the lysis of sensitive targets by these CTL in a time-dependent manner, i.e., the

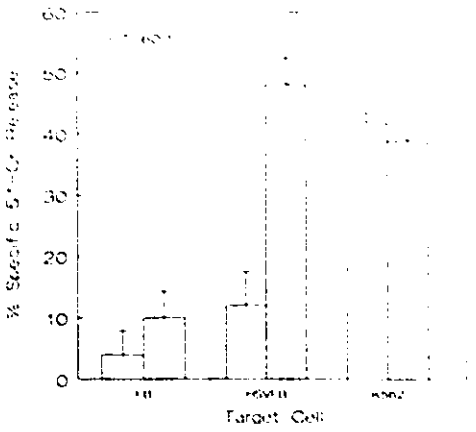

FIG. 3. HSV-FB are sensitive to ADCC. Human PBMC were isolated and tested for lytic activity against ^{51}Cr-labeled FB (FB), FB infected for 6 h with 10 PFU of HSV per cell (HSVFB), or K562 cells in the absence (fresh PBMC [□]) or presence of rabbit anti-HSV-1 immune serum (fresh PBMC plus anti-HSV [□]). Results shown are with an effector/target cell ratio of 60:1. Error bars represent standard deviation from the mean of triplicate wells.

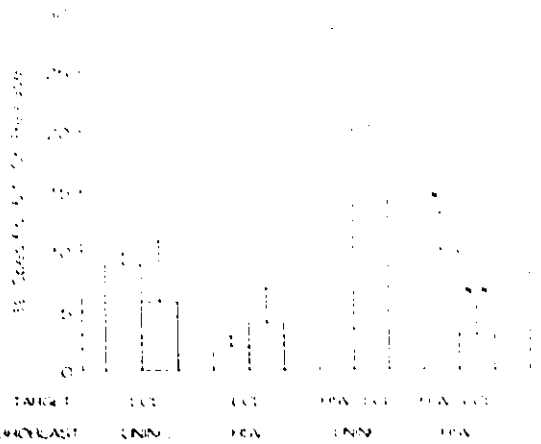

FIG. 5. Anti-HSV CTL are inhibited from lysis of sensitive targets when incubated on HSV-FB. Human primary anti-HSV CTL were generated and incubated for 2 h on uninfected skin FB or FB previously infected with 10 PFU of HSV for 20 h. After incubation, ^{51}Cr-labeled autologous uninfected LCL or LCL infected overnight with 10 PFU of HSV (HSV-LCL) were added in a 5-h ^{51}Cr release assay. Error bars represent standard deviation from the mean of triplicate wells. *, 61% inhibition of lysis of effectors at an effector/target cell ratio of 50:1 (□) compared with effectors incubated on uninfected FB ($P < 0.05$). **, 88% inhibition of lysis of effectors at an effector/target cell ratio of 25:1 (□) compared with effectors incubated on uninfected FB ($P < 0.05$).

longer the incubation of effectors with HSV-FB, the greater the inhibition (Fig. 6). The inhibition of alloantigen-specific CTL by HSV-FB relative to incubation on uninfected FB was 38, 58, and 79% at 2, 4, and 6 h, respectively ($P < 0.05$). Indeed, after 4 h of contact between alloantigen-specific CTL and HSV-FB, lysis of susceptible allogeneic targets was reduced to background levels seen with effectors treated with anti-CD3 (Fig. 6). The specificity of the alloantigen-specific CTL was shown by the marked lysis of allogeneic targets and the inability to lyse autologous labeled target cells (Fig. 6). These data suggest that the resistance of HSV-FB to lysis by human anti-HSV CTL and alloantigen-specific CTL was due to the ability of HSV-FB to inhibit the lytic capacity of CTL rather than an intrinsic resistance of HSV-FB to CTL-mediated lysis.

To further characterize the inhibition of CTL activity mediated by HSV-FB, we determined the time of infection of human FB required for inhibition of lytic activity to be observed. Human FB were infected for 2, 4, or 20 h with HSV before a 2-h exposure to alloantigen-specific CTL.

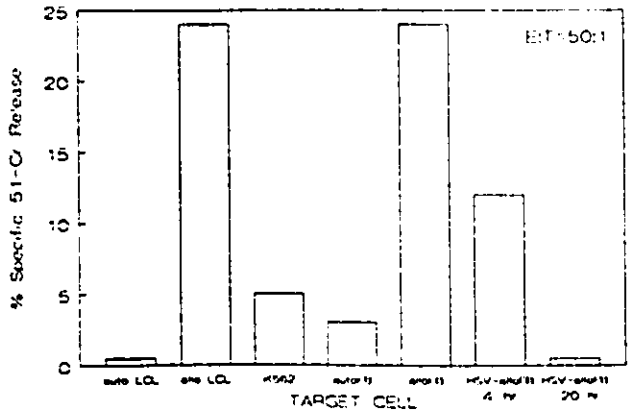

FIG. 4. Resistance of HSV-FB to alloantigen-specific CTL-mediated lysis increases with time. Alloantigen-specific CTL were generated, depleted of CD16$^+$ and CD56$^+$ cells by using anti-leu19 and anti-leu11b, respectively, and tested for lytic activity against ^{51}Cr-labeled K562 cells, autologous or allogeneic LCL, autologous or allogeneic FB, or allogeneic FB infected for 4 or 20 h with 10 PFU of HSV. Results shown are with an effector/target cell ratio of 50:1.

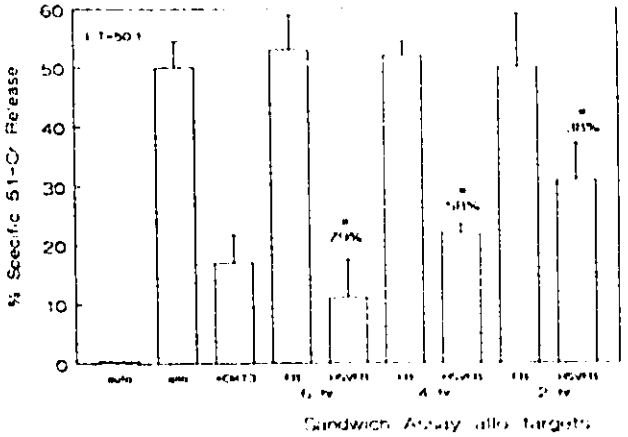

FIG. 6. Inhibition of lysis by alloantigen-specific CTL increases with increasing time of incubation of effectors with HSV-FB. Human alloantigen-specific CTL were generated and left untreated and tested for lytic activity against ^{51}Cr-labeled autologous LCL (auto) or allogeneic LCL (allo). Anti-CD3 (OKT3) was added to an additional group of effectors and allogeneic LCL (+OKT3). Effectors were also incubated on monolayers of FB or HSV-FB for 2, 4, or 6 h in a sandwich assay. After incubation, ^{51}Cr-labeled allogeneic LCL were added. Results shown are with an effector/target cell ratio of 50:1; error bars represent the standard deviation from the mean of quadruplicate wells. *, inhibition of alloantigen-specific CTL at 6, 4, and 2 h is 79, 58, and 38%, respectively, compared with lysis by alloantigen-specific CTL incubated on uninfected FB for the same time ($P < 0.05$).

TABLE 2. Inhibition of alloantigen-specific CTL lysis by FB infected for various times with HSV

Cell[a]	Expt 1[b]	% Inhibition	Expt 2[b]	% Inhibition[c]
auto LCL	5		0	
allo LCL	70		43	
+OKT3	16		11	
Sandwich assay[d]				
unFB	68		41	
HSV-FB				
2 h	41	40	15	63
4 h	38	44	15	63
20 h	48	29	28	32

[a] Target cells were allogeneic (allo) LCL except for autologous (auto) LCL control targets.

[b] Results shown are percent specific ^{51}Cr release at an effector/target cell ratio of 60:1 for experiments 1 and 2.

[c] Percent inhibition of alloantigen-specific CTL lysis by HSV-FB compared with lysis of alloantigen-specific CTL incubated on uninfected FB (unFB).

[d] Alloantigen-specific CTL were incubated for 2 h on uninfected FB or FB previously infected for 2, 4, or 20 h with HSV before the addition of ^{51}Cr-labeled allo LCL.

TABLE 3. Inhibition of alloantigen-specific CTL by HSV-FB is not mediated by a soluble factor[a]

Expt	auto[b]	allo	OKT3	Sandwich		Supernatant		Transwell	
				un	HSV	un	HSV	un	HSV
1	4	48	3	54	**23**[c]	54	54	—[d]	—
2	1	16	1	22	**9**	21	21	—	—
3	0	48	14	43	**13**	—	—	44	35
4	8	40	10	43	**10**	—	—	37	34

[a] To determine whether a soluble product was responsible for the inhibition of CTL activity, we determined lysis of ^{51}Cr-labeled allogeneic LCL after incubation of effector alloantigen-specific CTL for 4 h in a sandwich assay on uninfected FB (un) or FB infected for 20 h with HSV (HSV). Lysis of targets in a sandwich assay was compared with lysis mediated by alloantigen-specific CTL incubated for 4 h with filtered supernatant collected from uninfected FB or HSV-FB (experiments 1 and 2) or compared with lysis mediated by alloantigen-specific CTL incubated for 4 h in a 0.4-μm-pore-size Transwell exposed to uninfected FB or HSV-FB (experiments 3 and 4). Results shown are percent specific ^{51}Cr release at an effector/target cell ratio of 50:1 except for experiment 1, which was 60:1.

[b] Target cells were allogeneic (allo) LCL except for autologous (auto) control LCL targets.

[c] Boldface numbers are significantly different from cells incubated on uninfected FB ($P < 0.05$).

[d] —, not done.

Results demonstrated that by 2 h postinfection, human FB were able to profoundly inhibit the cytotoxic activity of alloantigen-specific CTL (Table 2). Indeed, FB infected for 2 and 4 h inhibited CTL activity to a greater extent than FB infected for 20 h (Table 2). These results are distinct from those reported by Confer et al. (5), who demonstrated that the degree of inhibition of NK- and LAK-cell lysis was dependent on the duration of HSV infection and was only manifest after 18 h of infection.

To determine whether the inhibition of CTL activity was specific for HSVs, we incubated alloantigen-specific CTL in sandwich assays with FB infected with HSV-1, HSV-2, vaccinia virus, or human adenovirus type 5. Results shown in Fig. 7 demonstrate that incubation of alloantigen-specific CTL for 4 h with FB infected with HSV-1 or HSV-2 significantly inhibited cytotoxic activity. Indeed, lysis was inhibited to levels observed after incubation of effector cells with anti-CD3. In contrast, incubation of alloantigen-specific CTL on FB left uninfected or infected with vaccinia virus or adenovirus type 5 did not inhibit lysis of relevant alloantigenic LCL target cells (Fig. 7). Thus, inactivation of alloantigen-specific CTL appears to be specific for HSV-FB.

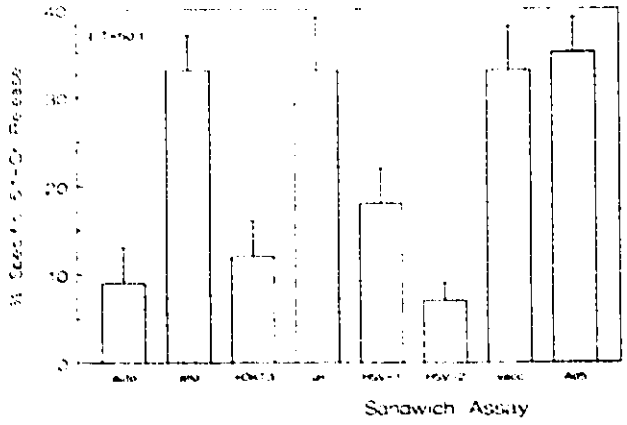

FIG. 7. Alloantigen-specific CTL are inactivated by HSV-FB but not FB infected with vaccinia virus or adenovirus. Human alloantigen-specific CTL were generated and left untreated and tested for lytic activity against ^{51}Cr-labeled autologous LCL (auto) or allogeneic LCL (allo). Anti-CD3 (OKT3) was added to an additional group of effectors and allogeneic LCL (+OKT3). Effectors were also incubated on monolayers of uninfected FB (un) or on FB infected for 20 h with HSV-1, HSV-2, vaccinia virus (vacc), or human adenovirus type 5 (Ad5) for 4 h in a sandwich assay. After incubation, ^{51}Cr-labeled allogeneic LCL were added. Results shown are with an effector/target cell ratio of 50:1; error bars represent the standard deviation from the mean of triplicate wells.

Inhibition of alloantigen-specific CTL is not mediated by a soluble factor. To determine whether a soluble product secreted from HSV-FB mediated the inhibitory effect on human CTL, we obtained cell-free supernatant from monolayers of uninfected FB or FB that were infected with HSV overnight. Supernatant was added to alloantigen-specific CTL for 4 h before the addition of ^{51}Cr-labeled sensitive target cells (allogeneic LCL). Results (Table 3, experiments 1 and 2) demonstrated that human alloantigen-specific CTL were not inhibited by a product present in the supernatant from HSV-FB. Indeed, lysis of allogeneic LCL was equivalent whether alloantigen-specific CTL were incubated with supernatant from uninfected FB or HSV-FB. In contrast, alloantigen-specific CTL-mediated lysis was significantly inhibited when the effector cells were incubated on HSV-FB in a sandwich assay (Table 3, experiments 1 and 2). Similar results were obtained when alloantigen-specific CTL were incubated for 2 or 6 h with supernatants from HSV-FB (data not shown).

To maximize exposure of effector CTL to FB supernatants, we used Transwell culture dishes. Each well of these culture dishes has a removable top chamber that is separated from the bottom chamber by a 0.4-μm-pore-size filter. Incubation of alloantigen-specific CTL in Transwell culture dishes that permitted exposure to FB supernatant from uninfected FB and HSV-FB without direct contact did not result in inhibition of cytotoxic activity (Table 3, experiments 3 and 4). In contrast, alloantigen-specific CTL were inhibited in sandwich assays when contact was permitted with HSV-FB but not with uninfected FB (Table 3). For both of these assays, the effectors were shown to be $CD3^+$ alloantigen-specific CTL since the effectors did not lyse

autologous labeled LCL and since lysis was inhibited in the presence of anti-CD3 antibody (Table 3). Although the data in Table 3 were generated with FB that had been infected overnight, FB infected with HSV for 2 or 4 h also did not inhibit alloantigen-specific CTL activity in a Transwell system (data not shown). Thus, the inhibitory effect mediated by HSV-FB on alloantigen-specific CTL does not appear to be mediated by a soluble factor but rather requires cell-to-cell contact.

DISCUSSION

In this study, we analyzed the recognition and lysis of HSV-FB by human anti-HSV CTL and alloantigen-specific CTL. In contrast to LCL, which were lysed by HLA-restricted anti-HSV CTL, at no time postinfection were HSV-FB lysed by human anti-HSV CTL. This resistance to antiviral CTL-mediated lysis was not due to a lack of infectivity of the target cells, since HSV-FB clearly expressed abundant amounts of HSV-specific proteins. Infection of FB with HSV also resulted in a time-dependent resistance to alloantigen-specific CTL-mediated lysis. Alloantigen-specific CTL lysis of FB infected for 4 h with HSV was reduced relative to the lysis of uninfected allogeneic FB, and infection for 20 h rendered the cells fully resistant to lysis. The apparent resistance of HSV-FB to human CTL was reminiscent of studies reported by Confer et al. (5), who demonstrated that human FB infected overnight with HSV were not lysed by human NK or LAK cells. The mechanism of resistance of HSV-FB to human NK- and LAK-cell lysis was shown to be due to disarming of the killer cells by direct contact with HSV-FB (5). Here we extend the results of Confer et al. (5) and demonstrate that incubation of anti-HSV CTL or alloantigen-specific CTL with HSV-FB for 2 to 6 h rendered the CTL incapable of lysing their normally sensitive target cells. Indeed, by only 2 h postinfection with HSV, human FB were able to profoundly inhibit the cytotoxic activity of alloantigen-specific CTL. This effect appeared to be HSV specific since infection of FB with HSV-1 or HSV-2 resulted in CTL inactivation, whereas infection of FB with adenovirus or vaccinia virus did not diminish CTL activity. Last, the inhibition of CTL by HSV-FB is not mediated by a soluble factor, but rather requires cell-to-cell contact. Thus, HSV-FB are not inherently resistant to lysis by anti-HSV CTL or alloantigen-specific CTL, but rather contact of CTL with HSV-FB resulted in inactivation of the CTL.

Surprisingly, the first evidence that HLA-restricted HSV-specific CTL-mediated cytotoxicity could be demonstrated in humans was shown using skin FB cultures from HLA-typed individuals (25). Subsequently, though, studies of human anti-HSV CTL have relied heavily on the use of autologous LCL as target cells (30–35). Clearly, this was due to the need for HLA-matched target cells and the ease with which LCL can be generated from the peripheral blood of individuals. Since LCL express both class I and II HLA molecules, it was shown that humans have two distinct populations of anti-HSV CTL (24, 30, 32). One is $CD4^-$ $CD8^+$ and restricted by HLA class I, and the other is $CD4^+$ $CD8^-$ and restricted by HLA class II. Activation of both $CD4^+$ and $CD8^+$ CTL by stimulation with UV-inactivated HSV was confirmed by using HSV-infected human monocytes and macrophages as targets (27). Although two subsets of anti-HSV CTL exist in humans, their specificity and role in HSV infections are not well understood.

Studies performed by Yasukawa et al. (30) demonstrated that HLA class II-restricted $CD4^+$ CTL were mainly induced by stimulating peripheral blood lymphocytes with UV-inactivated cell-free HSV antigen, whereas HLA class I-restricted $CD8^+$ CTL were induced by culturing peripheral blood lymphocytes with autologous FB that were absorbed with UV-inactivated HSV. Further, in contrast to our findings, they demonstrated that the $CD8^+$ CTL generated after stimulation with HSV-pulsed autologous FB were able to kill HSV-infected autologous FB (30). Similar results demonstrating the ability of virus-infected human FB to stimulate class I HLA-restricted CTL were reported for human cytomegalovirus (4) and varicella-zoster virus (9).

We were unable to confirm the findings of Yasukawa et al. (30). Indeed, we found that HSV-FB were resistant to $CD8^+$ anti-HSV CTL. This resistance was not due to a lack of infectivity or viral protein production. Human FB infected with HSV expressed abundant levels of HSV-specific proteins as demonstrated by immunoprecipitation, immunofluorescence, and their susceptibility to ADCC in the presence of anti-HSV serum. Although human FB were infectible with HSV, expression of viral proteins does not guarantee the susceptibility of target cells to CTL-mediated lysis. We are now well aware that CTL recognize processed fragments of viral proteins selectively bound by MHC molecules (28). For appropriate antigen presentation to occur, viral proteins must be properly degraded to peptides in the cytoplasm and transported into the endoplasmic reticulum and selectively interact with a folding MHC molecule. Appropriately folded molecules with their bound peptides are then transported to the cell surface for recognition by appropriate CTL. Although we did not pursue problems of HSV antigen processing or presentation in infected human FB, our results indicate that the resistance of HSV-FB to human CTL is a consequence of inactivation of cytotoxic activity by HSV-FB.

Initial studies by Fitzgerald et al. (7) demonstrated that acutely infected human FB were sensitive to human NK-cell activity. Subsequently, though, they found that FB infected for longer periods (20 h) were markedly less susceptible to NK-cell lysis (8). These observations were extended by Confer et al. (5), who demonstrated that human FB and endothelial cells became progressively less susceptible to NK and LAK cell-mediated lysis with duration of HSV infection. Using sandwich assays in which NK or LAK cells were placed atop a monolayer of HSV-FB for variable times before the addition of labeled, susceptible target cells, they demonstrated that human FB infected with HSV potently inhibited the lytic activity of NK and LAK cells.

Our results further extend those of Confer et al. (5) and clearly demonstrate that human FB infected with HSV are also able to potently inhibit the lytic activity of both MHC-restricted $CD3^+$ anti-HSV CTL and alloantigen-specific CTL. Indeed, exposure of anti-HSV CTL to HSV-FB resulted in the inhibition of both $CD4^+$ and $CD8^+$ HLA-restricted anti-HSV CTL since lysis of HSV-infected LCL target cells was abrogated. It is likely that cytolytic cells with any specificity will be inactivated by HSV-FB. Interestingly, the kinetics of infection of human FB needed to inhibit alloantigen-specific CTL differed from that observed with NK and LAK cells. While at least 8 h of FB infection were required to observe inhibition of NK- and LAK-cell lysis which increased with duration of infection, human FB infected for only 2 hours were able to profoundly inhibit alloantigen-specific CTL. Indeed, FB infected for 2 and 4 h inhibited CTL activity to a greater extent than FB infected for 20 h.

Confer et al. (5) referred to the ability of HSV-FB to inhibit killer lymphocytes as "disarming." We have elected not to use this term until we better understand the mechanism of the inhibition. Disarming implies a loss of cytolytic machinery. In preliminary experiments (18), we have found that CTL that were incubated with HSV-FB could subsequently mediate antibody-redirected cytolysis of target cells bearing Fc receptors and incubated with anti-CD3 antibodies. These results imply that CTL still have intact cytolytic machinery but are inhibited from killing susceptible targets. Further, we have found that CTL are inactivated for at least 20 h, but we do not know the duration of inhibition nor whether the effect is transient or results in long-term ablation of lytic activity.

Soluble products released from certain cell types have been shown to inhibit the proliferation of T cells and the lytic phase of human CTL. Transforming growth factor β, produced from an HSV-2-induced murine tumor cell line, was shown to suppress proliferative responses in a mixed lymphocyte reaction with human PBMC (19). Additionally, a 20- to 30-kDa molecule released from $CD8^+$ $CD57^+$ lymphocytes obtained from AIDS patients has been shown to inhibit the effector phase of MHC-restricted CTL (23). Recently, it was also shown that supernatants generated by incubating human PBMC with HSV-FB contain tumor necrosis factor (13). In the present study, the filtered supernatant collected from HSV-FB did not mediate inhibition of alloantigen-specific CTL. Further, incubation of CTL in Transwell dishes that permitted exposure to FB supernatant from HSV-FB without direct contact did not result in inhibition of cytotoxic activity. Conditioned medium from HSV-FB was also shown to have no effect on the lytic capability of NK or LAK cells (5). Thus, the inhibitory effect mediated by HSV-FB is not mediated by a soluble factor, but rather requires direct cell-to-cell contact.

Since direct contact with HSV-FB is required for the inhibition of cell-mediated cytotoxicity, it is natural to suspect that a surface-expressed molecule on infected cells is responsible. Confer et al. (5) demonstrated that HSV-FB incubated for 18 h in the presence of tunicamycin, an inhibitor of N-linked glycosylation, lost the capacity to inhibit NK cell-mediated cytolysis. They interpreted their results as suggesting that surface expression of HSV glycoproteins was involved in suppressing the cytotoxic cells. We are currently investigating the role of viral glycoproteins in mediating the inhibitory ability of HSV-FB on CTL-mediated lysis.

Hommel-Berrey et al. (10) have described a contact-dependent mechanism of CTL inactivation by an Epstein-Barr virus-transformed cell line, PAMO, and suggested that it is due to the modulation of a key membrane molecule (e.g., T-cell receptor/CD3 complex, CD4 or CD8 and class I MHC) and the lack of certain secondary messengers involved in signal transduction (10). Taken together with our results and those of Confer et al. (5), these observations imply the existence of a signal transduction pathway in all cytolytic cells whose activation inhibits lytic activity.

The inactivation of human anti-HSV CTL may have important implications in HSV infections. Immunocompromised patients with defects in cell-mediated immunity experience more severe and extensive HSV infections and reactivations than those with deficits in humoral immunity (6). During normal HSV reactivations, suppression of certain immune responses has been detected. For example, immediately after the onset of an HSV-1 lesion, the in vitro production of interleukin-2 and alpha interferon from PBMC stimulated with HSV-1 antigen was suppressed relative to the production of these cytokines from PBMC isolated during the convalescent phase (4 to 14 days after lesion onset) (11). Similarly, cell-mediated cytotoxicity, most likely NK-cell lysis, was also significantly lower in patients in the recrudescent phase relative to the convalescent phase (11). Preliminary data from our laboratory demonstrate that in two donors with frequent HSV recurrences, HSV-specific CTL were undetectable 0 to 5 days before the onset of a lesion, whereas 7 days after the onset, anti-HSV CTL could be measured (18). Taken together, these findings suggest that suppression of CTL activity and other immune parameters is coincident with the onset of an HSV lesion, implying that the virus itself is responsible for a transient immunosuppression leading to viral reactivation. Possibly, infection of FB and endothelial cells with HSV may result in a transient local suppression of cell-mediated immune responses which allows the virus to spread, form a lesion, and infect other ganglia. This could account for the recurrent episodes and persistence of HSV in immunocompetent individuals.

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CHAPTER 5

INHIBITION OF HUMAN CTL-MEDIATED LYSIS BY FIBROBLASTS INFECTED WITH HERPES SIMPLEX VIRUS

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Running Head: HSV-FB inhibit human CTL-mediated lysis

ABSTRACT

Previously, we demonstrated that human anti-HSV CTL and allo-antigen specific CTL were inhibited in lysing their normally sensitive target cells when they were exposed to human fibroblasts (FB) infected with herpes simplex virus (HSV). In this study, the mechanism of inhibition of CTL lytic function by FB infected with HSV-1 (HSV-FB) was studied. CTL exposed to HSV-FB early (2 hours) in the infectious cycle were inhibited by a mechanism that appears to be distinct from the inhibition of lytic function mediated by HSV-FB at late times (20 hours) during the infection cycle. The inhibition of CTL-mediated lysis by FB infected with HSV-1 for 2 hours required the expression of ICP4, an immediate-early protein of HSV-1, but not the production of infectious virus or virus-induced shut-off of host protein synthesis. In contrast, the expression of HSV-specific glycoproteins essential for viral infectivity (glycoproteins B, D, H, K and L), and thus, infectious virus, was required for inhibition of CTL lytic function by FB infected with HSV-1 for 20 hours. Further, CTL exposed to FB infected with HSV-1 for 20 hours expressed HSV-specific proteins indicating that they were infected with HSV-1. Cell-to-cell spread of HSV-1 appeared to be the major mode of transmission because (1) an insufficient level of HSV-1 was present in the supernatant of HSV-FB to inhibit CTL lytic function, and (2) paraformaldehyde-fixed HSV-FB did not inhibit CTL-mediated lysis. The inhibition of CTL lytic function by HSV-FB may be an important mechanism of HSV-induced immunosuppression permitting the virus to spread and persist in immunocompetent hosts after primary infection or reactivation of latent HSV.

INTRODUCTION

In order for viruses to persist in their hosts, they require mechanisms of avoiding the host's immune defences. Herpes simplex viruses (HSV-1 and HSV-2) have evolved numerous mechanisms of avoiding humoral and cellular immune responses allowing them to establish persistent life-long infections (reviewed in 1). The most common mechanism of immune evasion by herpesviruses is the establishment of latency and restricted viral gene expression allowing the virus to hide from the immune system (2). HSV remains latent in neural cells providing it with an environment capable of avoiding cell-mediated immune recognition since neural cells do not express MHC molecules (3), proteins required for presentation of viral peptides to T cells.

Upon reactivation of latent HSV, viral genomes are transported to dermal and epidermal surfaces where virus replication and subsequent release of progeny virus occurs. During viral replication, expression of viral gene products renders the virus susceptible to recognition by the immune system. Another mechanism of HSV immune evasion involves the expression of a virally-encoded Fc receptor (FcR) (glycoproteins E and I) found on the surface of infected cells (4). HSV-FcR is thought to participate in antibody bipolar bridging reducing the effectiveness of antibody-dependent cellular cytotoxicity (ADCC) (5). Down-regulation of MHC class I molecules by HSV, preventing T cell recognition of infected cells, has also been demonstrated (6).

Non-specific immune mechanisms have been shown to be directly inhibited

by HSV-1. Infection of monocytes with HSV inhibited presentation of antigen to resting T cells and reduced the production of IL-1α and TNF-β after phorbol-ionomycin stimulation (7). NK cell and lymphokine-activated killer (LAK) cell lytic function was inhibited when cells were exposed to FB or endothelial cells infected with HSV-1 (8).

Recently it has been demonstrated that human FB infected with HSV (HSV-FB) are resistant to lysis by anti-HSV CTL, allo-antigen specific CTL (allo-CTL) (9) and human CTL clones (10). We further showed that HSV-FB are not only resistant to lysis by CTL but can also inhibit CTL lytic function (9). Human anti-HSV-1 CTL and allo-CTL were inhibited from lysing normally sensitive target cells when CTL were incubated with HSV-FB (9). CTL lytic function could be inhibited by FB infected with HSV-1 for as little as 2 hours. HSV-FB inhibition of CTL lytic function was dependent on cell-to-cell contact and did not involve the production of a soluble factor from the infected FB (9).

In this report, the mechanism of inhibition of CTL lytic function by HSV-FB was studied. It appears that two distinct inhibitory mechanisms are involved. The first involved FB infected with HSV-1 for as little as 2 hours and required the expression of ICP4 but not infectious virus or virus-induced shut-off of host protein synthesis. The second mechanism occurred later in the HSV replication cycle and involved the cell-to-cell spread of HSV-1 from FB infected with HSV-1 for 20 hours to the CTL rendering the CTL inactive. The elucidation of mechanisms involved in HSV-induced immunosuppression may foster the development of preventative

or therapeutic strategies aimed at controlling these pathogens in humans.

MATERIALS AND METHODS

Cell lines. Human EBV-transformed B lymphoblastoid cell lines (LCL) and human FB cell lines were established as previously described (9). LCL were maintained in RPMI 1640 containing 10% FCS, 102 U of penicillin G per ml, 10 μg of streptomycin sulfate per ml, 2 mM l-glutamine and 10 mM HEPES (hereafter termed complete media). FB and Vero cells were maintained in α-MEM containing 10% FCS with supplements mentioned above.

Viruses. The following viruses were used: HSV-1 strain F (obtained from P. Spear, University of Chicago), HSV-2 strain 333, K082 (gB-) (11), MP4 (gC-) (12), F-Us6Kan (gD-) (13), In1404 (gE-) (4), SCgHZ (gH-) (14), F-Us7Kan (gI-) (4), FgKβ (gK-) (L. Hutchinson and D.C. Johnson, unpublished observations), KOSgLβ (gL-) (15) (all kindly provided by D. Johnson, McMaster University), HSV-1 strain PAA^R5, vhsA and vhsB (viruses with disruptions in the UL41 gene of PAA^R5) (16), and d120 (HSV-1 mutant with a 4.1 kb deletion in the ICP4 gene) (17) (all kindly provided by J. Smiley, McMaster University).

Cytotoxic cells. Human alloantigen-specific CTL were prepared in a one-way mixed lymphocyte reaction as previously described (9). Human $CD8^+$ HLA-A2 specific CTL clone, 3G5, was kindly provided by S. Riddell, Fred Hutchinson Cancer Research Institute, Seattle (18).

CTL-mediated cytotoxicity assays. Standard chromium release assays were

used to assess CTL-mediated cytotoxicity. Autologous and allogeneic LCL, used as target cells, were labeled with 200 μCi of $Na_2{}^{51}CrO_4$ (New England Nuclear Corp., Boston, MA) for 1.5 hours, washed and counted. Target cells (1 x 10^4/well for bulk allo-CTL, 5 x 10^4/well for 3G5) were added to effectors at various E:T ratios in 96 well flat-bottomed microtiter plates (Nunclon, Roskilde, Denmark) and incubated for 4-5 hours at 37°C in 5% CO_2. Target cells were also cultured in media alone (spontaneous release) or with 1 N HCL (maximal release). After 5 hours of incubation, 100 μl of supernatant was removed from each well and counted in a gamma counter. The percent specific ^{51}Cr-release was calculated as follows:

$$\frac{\text{cpm experimental release - cpm spontaneous release}}{\text{cpm maximal release - cpm spontaneous release}} \times 100\%$$

The spontaneous release was always less than 30% of the maximal release.

Sandwich assays were performed as previously described (9). Briefly, uninfected FB and FB infected for various times with 10 PFU/cell of wild-type HSV or an HSV mutant virus were trypsinized and 1x10^4 cells/well added to 96-well flat-bottomed plates. FB were allowed to recover and adhere for 1-2 hr. Following adherance, effectors were added to the FB for 2-3 hours prior to addition of ^{51}Cr-labeled target cells in a ^{51}Cr-release assay. In some cases, HSV-FB were fixed with 1% paraformaldehyde for 10 minutes and washed 3 times with complete media prior to use in a sandwich assay.

Separation of CTL from HSV-FB and detection of HSV-specific proteins in CTL. CTL were incubated for 2 hours with uninfected FB or FB infected with HSV-

1 for 2 or 20 hours in 60 mm tissue culture dishes in 1.5 ml. Unattached CTL were removed from FB monolayers (removed CTL). The remaining cells were removed by 0.5% trypsin in EDTA and 3 ml complete media was added to inactivate the trypsin (trypsinized CTL). Discontinuous Percoll gradients were used to separate CTL from FB. Percoll (90% percoll, 9% 10X HBSS, 1% HEPES, pH 7.4) was diluted to 40% and 70% with complete media and 3 mls of 70% percoll solution was underlayed beneath 7 mls of 40% percoll solution in 15 ml conical centrifuge tubes. Removed CTL or trypsinized CTL were layered onto percoll gradients and centrifuged at 1700 rpm for 30 minutes. The lymphocytes migrated to the interface between the 40% and 70% percoll and the FB remained on top of the 40% percoll. The CTL fraction was removed and washed 2 times with 10 mls PBS. Cells were washed 2 times with 10 ml media 199 lacking methionine (199-meth) and resuspended in 1 ml 199-meth containing 500 μCi of ^{35}S-methionine (New England Nuclear). Following 1.5 hours incubation at 37°C, cells were washed 3 times with 10 ml cold PBS. Radioimmunoprecipitation assay (RIPA) buffer (1.1 ml) (150 mM NaCl, 50 mM Tris [pH 7.2], 0.1% SDS, 1% Triton X-100 and 1% Na deoxycholate) containing 1 mM PMSF, 1 mg/ml BSA and 100 KIU aprotonin (Sigma Chemical Co., St. Louis, MO) was added to the cell pellet and cell lysates were spun in a microfuge for 15 minutes at 4°C. Supernatant was removed and frozen at -70°C. HSV-specific proteins were immunoprecipitated from cell extracts with protein A-Sepharose (Pharmacia Chemicals, Dorval, Quebec, Canada) and 58S (monoclonal antibody specific for ICP4) or 15βB2

(monoclonal antibody specific for glycoprotein B) (both kindly provided by D. Johnson, McMaster University) (19) for 3 hours at 4°C with constant rotation. Immunoprecipitates were washed 4 times with 1 ml cold RIPA buffer, 50 µl sample buffer (containing 2-ME at 1:50) was added, and samples heated at 95°C for 5 min. Samples (40 µl) were loaded onto a continuous 10% polyacrylamide gel and electrophoresed at 65 V overnight. Gels were fixed in water-methanol-acetic acid (40:50:10) for 1 hour, infused with Enlightning (New England Nuclear Corp.) for 30 min., dried, and exposed to Kodak XAR film.

Titre of HSV-1 in supernatant from HSV-FB. FB infected for various times with 10 PFU/cell HSV-1 were trypsinized and $1x10^4$ cells/well in 250 µl complete media were added to 96-well flat-bottomed plates. Following adherance, supernatant from 8 wells was removed immediately or 3 hours later to represent the times of the addition of CTL or target cells, respectively, in a sandwich assay. Supernatant was centrifuged at 1200 rpm for 5 minutes to remove any cells and frozen at -70°C. Supernatant was serially diluted in α-MEM and 0.8 ml added to Vero cell monolayers in 6-well tissue culture plates for 2 hours. Cells were overlayed with 2 ml α-MEM containing 10% FCS and 0.05% human immue serum (Canadian Red Cross Society, Ottawa) and incubated for 72 hours at 37°C. To view viral plaques, supernatant was removed from the wells and cells were stained with crystal violet.

Statistical analysis. Results were analysed by a student's two-tailed t test using Instat statistical software. A p-value <0.05 was taken to be significant.

RESULTS

Role of virus-induced host shut-off in HSV-FB mediated inhibition of CTL lytic activity. A consequence of infection by HSV-1 is the shut-off of host protein synthesis by a virion component, vhs, encoded by the UL41 gene (16). To determine if shut-off of a host cell protein by HSV-1 was involved in the inhibition of CTL lytic function by HSV-FB, FB were infected with vhsA or vhsB, two viruses with disruptions in the UL41 gene. Figure 1 demonstrates that allo-CTL incubated with FB infected for 2 or 20 hours with HSV-1, PAA^R5 (the parent virus to mutants vhsA and vhsB), vhsA or vhsB were inhibited in lysing normally sensitive target cells. In contrast, allo-CTL incubated on uninfected FB clearly lysed allo LCL (Figure 1). These results demonstrate that HSV-induced shut-off of a host cell protein is not involved in the inhibition of CTL lytic activity by HSV-FB.

Role of ICP4 in the inhibition of CTL-mediated lysis by HSV-FB. Since inhibition of CTL-mediated lysis was observed following exposure to HSV-FB following only 2 hours of infection, we analysed the role of HSV immediate-early proteins. To determine the role of ICP4, an immediate-early protein of HSV-1 involved in transactivating other viral genes, in the inhibition of CTL lytic activity by HSV-FB, FB were infected with d120 for 2 or 20 hours prior to use in a sandwich assay. d120 is a deletion mutant of HSV-1 which encodes truncated forms of ICP4 (17). Exposure of allo-CTL (Figure 2A) or cloned 3G5 cytotoxic T cells (Figure 2B) to FB infected for 2 or 20 hours with HSV-1 profoundly inhibited their lytic activity. In contrast, allo-CTL and 3G5 cells retained lytic activity after being

exposed to uninfected FB or FB infected for 2 or 20 hours with d120 (Figure 2). These results suggest that ICP4 may be directly involved in the inhibition of CTL-mediated lysis by HSV-FB or indirectly involved by the transactivation of other HSV-1 proteins, such as HSV glycoproteins.

Role of HSV-specific glycoproteins in the inhibition of CTL-mediated lysis. The role of HSV-1 glycoproteins in the inhibition CTL lytic function by HSV-FB was studied by infecting FB with viruses mutant in individual HSV-1 glycoprotein genes. Figure 3 demonstrates that FB infected for 20 hours with HSV-1 or HSV-2, but not uninfected FB, significantly inhibited allo-CTL lytic activity. Allo-CTL lytic activity was also inhibited when allo-CTL were exposed to FB infected with viruses mutant in gC, gE or gI. In contrast, FB infected with viruses mutant in gB, gD, gH, gK and gL, did not inhibit CTL-mediated lysis (Figure 3). Each glycoprotein mutant caused CPE in the FB (data not shown) indicating that the FB were infected. These results demonstrate that gC, gE and gI, which are dispensable for viral infectivity (20), were not involved in mediating inhibition of CTL-mediated lysis by HSV-FB infected for 20 hours. However, lack of any one of the HSV glycoprotein genes essential for viral infectivity (gB, gD, gH, gK and gL) (20,15, L. Hutchinson and D.C. Johnson, personal communication) abrogated the inhibition of CTL-mediated lysis by FB infected with HSV-1 for 20 hours. These results suggest that the production of infectious virus from FB infected with HSV-1 for 20 hours was required to inhibit CTL-mediated lysis

To determine if the production of infectious virus from FB infected with HSV-

1 for 2 hours was necessary to inhibit the lytic activity of allo-CTL. FB were infected with F-UsKan6, a gD minus virus or KOSgLβ, a gL minus virus, that can infect cells but not produce infectious virus (13,15). Allo-CTL lytic activity was significantly inhibited when allo-CTL were exposed to FB infected with HSV-1, gD- or gL- viruses (Figure 4). These results suggest that the production of infectious virus was not required for the inhibition of CTL lytic activity by FB infected with HSV-1 for 2 hours and indicate that FB infected for 2 hours inhibit allo-CTL lytic activity by a mechanism distinct from FB infected for 20 hours.

Expression of HSV-specific proteins in a cloned CTL cell line exposed to HSV-FB. To determine if CTL exposed to HSV-FB became infected with HSV-1, the expression of HSV-specific proteins was measured in cloned 3G5 cells exposed to FB previously infected for 2 or 20 hours with HSV-1. Unattached 3G5 were removed directly from HSV-FB (removed) and the remaining CTL were trypsinized and separated from the FB using percoll gradients (trypsinized). Figure 5 demonstrates that 3G5 exposed for 3 hours to uninfected FB or FB that had been infected with HSV-1 for 2 hours did not express detectable levels of ICP4 or gB. When FB infected for 2 hours were removed from the 3G5 cells after a sandwich assay (5 hours total infection time), they expressed abundant levels of ICP4 and gB (data not shown), indicating that the Percoll gradient efficiently separated the HSV-FB and the CTL. In contrast, 3G5 exposed for 3 hours to FB that had been infected with HSV-1 for 20 hours expressed ICP4 in both the removed and trypsinized fractions (Figure 5). The expression of gB was detected

in 3G5 trypsinized from FB infected for 20 hours with HSV-1 (Figure 5). These results demonstrate that CTL exposed for 3 hours to FB previously infected with HSV-1 for 20 hours became infected and expressed viral gene products. In contrast, 3G5 exposed to FB infected with HSV-1 for 2 hours did not become infected as measured by viral protein expression.

Direct infection of CTL by HSV-1. Although CTL exposed to FB infected with HSV-1 for 20 hours expressed viral proteins and were inhibited in lysing normally sensitive target cells, it is not clear whether infection of CTL by HSV-1 was the mechanism of inhibition of lytic activity. Therefore, allo-CTL were directly infected with HSV-1 to determine if HSV-infected allo-CTL were inhibited in lysing target cells. Figure 6 demonstrates that the infection of allo-CTL for 2 hours with HSV-1 at MOIs of 10 or less had no significant effect on CTL lytic function ($p>0.05$). However, when allo-CTL were infected with 100 PFU/cell of HSV-1, CTL lytic activity was inhibited by 75% ($p<0.05$) (Figure 6). Therefore, allo-CTL were susceptible to direct infection with HSV-1, and at a high MOI, HSV-1 can inhibit CTL-mediated lysis at 2 hours post-infection.

Titre of HSV-1 in supernatant of HSV-FB. Cell-free virus was measured in the supernatant of HSV-FB at times corresponding to the addition of CTL or the addition of ^{51}Cr-labeled target cells to determine if levels of virus released from HSV-FB were high enough to inhibit CTL lytic activity. Table I demonstrates that low levels of virus, representing .001 to .003 PFU/CTL, were present in the supernatant of FB infected for 2 hours with HSV-1, levels that are too low to inhibit

CTL lytic activity. Levels of virus increased to 8 and 10 PFU/CTL when FB were infected for 20 hours with HSV-1 (Table I). This indicates that a significant level of virus was present at the time CTL were added to FB previously infected with HSV-1 for 20 hours although this level was not high enough to significantly inhibit allo-CTL lytic activity (Figure 6). Although it may partly contribute to the inhibition, direct infection of CTL by cell-free virus produced by FB infected with HSV-1 for 20 hours does not account for the degree of inhibition achieved in a sandwich assay

Cell-to-cell spread of HSV-1 from FB infected for 20 hours. To determine if cell-to-cell spread of HSV-1 from FB infected for 20 hours to allo-CTL was the mode of viral transmission, HSV-FB were fixed with paraformaldehyde to prevent cell-to-cell spread of virus. Figure 7 demonstrates that incubation of allo-CTL with FB infected with HSV-1 for 20 hours significantly inhibited their lytic activity. In contrast, exposure of allo-CTL to fixed HSV-FB did not inhibit allo-CTL lytic activity (Figure 7). These results suggest that cell-to-cell spread of HSV-1 from HSV-FB, infected for 20 hours, to allo-CTL was required for the inhibition of CTL-mediated lysis.

DISCUSSION

The exposure of human CTL to FB infected with HSV profoundly inhibits their ability to lyse normally sensitive target cells. Our results suggest that FB infected with HSV-1 for 2 hours inhibited CTL-mediated lysis by a mechanism distinct from the inhibition of lytic function mediated by FB infected with HSV-1 for 20 hours. The expression of essential HSV glycoproteins and thus, the production of infectious virus, was necessary for FB infected for 20 hours to inhibit CTL lytic function. CTL exposed to these HSV-FB expressed HSV-specific proteins indicating that they were infected. Cell-to-cell spread of HSV-1 from the infected FB to the CTL appeared to be the principal mode of viral transmission since (1) supernatant from HSV-FB did not inhibit CTL lytic function (9), (2) the virus titre in the supernatant of HSV-FB was too low to provide the level of inhibition achieved in a sandwich assay, and (3) fixed HSV-FB did not inhibit CTL lytic function. Inhibition of CTL-mediated lysis by FB infected with HSV-1 for 2 hours was dependent on the expression of ICP4 but not on the production of infectious virus from the infected FB or virus-induced shut-off of host protein synthesis. Further, CTL exposed to FB infected with HSV-1 for 2 hours did not express HSV-specific proteins indicating that viral infection of CTL was not necessary to inhibit their lytic ability. Thus, two distinct mechanisms of inhibition of CTL-mediated lysis appear to be involved: one that occurred early and one that occurred late in the HSV-1 infection cycle.

We have previously shown that human CTL exposed to HSV-FB are

profoundly inhibited in lysing normally sensitive target cells by a contact-dependent mechanism (9). The inhibition of CTL lytic activity by HSV-FB occurred as early as 2 hours post-infection suggesting that an event occurring early in HSV-1 infection was involved in this inhibition. HSV immediate-early or α proteins are the first proteins produced upon virus infection. ICP4 (α4, IE175) is essential for viral replication and is required for transcription of β (early) and γ (late) genes and for autoregulation (repression) of its own synthesis (20). d120 is a deletion mutant of HSV-1 which induces the synthesis of truncated forms of the ICP4 polypeptide (17). Upon infection with d120, only ICP0 (α), ICP27 (α), ICP22 (α) and ICP6 (β) proteins are expressed (17). Further, early and late gene expression is inhibited as is viral DNA replication (17). Our results demonstrated that the inhibition of CTL-mediated lysis by HSV-FB at early and late times required the expression of ICP4.

HSV glycoproteins require the expression of ICP4 and other immediate-early proteins (20). Further, HSV glycoproteins are expressed on the surface of HSV-infected cells and cell-to-cell contact was necessary for HSV-FB to inhibit CTL-mediated lysis (9). Also, Confer et al. (8) showed that tunicamycin treatment of HSV-FB prevented their ability to inhibit NK and LAK cell lytic function, implicating a role for glycoproteins in mediating the inhibitory phenomenon. Therefore, FB were infected with viruses mutant in glycoproteins which are essential (gB, gD, gH, gK, gL) or non-essential (gC, gE or gI) for viral infectivity and multiplication in cell culture (20,15, L. Hutchinson and D.C. Johnson, personal communication).

Although viruses mutant in essential glycoprotein genes can infect cells and cause CPE, no infectious virus is produced (20,15). In contrast, cells infected with viruses mutant in non-essential glycoprotein genes can replicate and produce infectious virus.

Our results demonstrate that lack of any one of the glycoproteins involved in infectivity abrogated the inhibition of CTL-mediated lysis by FB infected with HSV-1 for 20 hours. Expression of non-essential HSV glycoproteins was not required for inhibition. These results indicate that the production of infectious virus from FB infected with HSV-1 for 20 hours was necessary for inhibition of CTL lytic function. CTL exposed to FB infected with HSV-1 for 20 hours expressed HSV-specific proteins indicating that they were infected. Although the direct infection of CTL with high titres of cell-free HSV-1 inhibited CTL-mediated lysis, an insufficient amount of HSV-1 was present in the supernatant of HSV-FB to account for the level of inhibition in a sandwich assay. The inhibition of CTL-mediated lysis by direct infection required 100 PFU/cell HSV-1, a level that is 10 times higher than that present in supernatant from FB infected with HSV-1 for 20 hours. These results suggested that the mode of transmission of HSV-1 from FB infected with HSV-1 for 20 hours was via cell-to-cell spread of HSV-1 from the infected FB to the CTL. This was confirmed using fixed HSV-FB which did not inhibit CTL-mediated lysis. Therefore, the mechanism of inhibition of CTL lytic function by FB infected with HSV-1 for 20 hours appeared to be by cell-to-cell spread of virus. Cell-to-cell spread of virus may be the natural mode of infection in vivo since HSV

infection can occur in the presence of neutralizing antibodies (21).

The inhibition of CTL lytic activity by FB infected with HSV-1 for 2 hours required the expression of ICP4 but not a cellular protein shut-off by vhs nor the production of infectious virus. Further, CTL exposed to FB infected with HSV-1 for 2 hours did not express HSV-specific proteins suggesting that infection of CTL was not necessary to inhibit CTL lytic function. Because inhibition requires cell-to-cell contact, a cell-surface molecule may be involved in the inhibition of CTL lytic activity by exposure to FB infected with HSV-1 for 2 hours.

Although HSV binds to resting and activated B and T lymphocytes, viral replication has been detected in activated lymphocytes only (22,23). The innate resistance of freshly isolated, or resting, lymphocytes may be due to the HSV-induced expression of two uncharacterized cellular proteins which are not induced in activated lymphocytes (24). The permissiveness of activated lymphocytes to HSV infection may be due to the upregulation of HSV receptors as a result of stimulation with mitogens (22) or antigen. While these studies demonstrate that activated lymphocytes can act as targets for HSV replication, the effect of HSV infection on lymphocyte function has not been well investigated. In the present study, we demonstrate that CTL lytic function was profoundly inhibited when CTL were infected by cell-to-cell spread of HSV-1 from infected FB or by direct infection with high titres of HSV. HSV infection not only disrupts CTL function but monocyte accessory cell function as well. Overnight culture of newborns' monocytes with HSV inhibited the presentation of staphylococcal enterotoxin to resting T cells as

well as decreasing the production of IL-1α and TNF-β in response to phorbol-ionomycin stimulation (7). Innate immune mechanisms are also sensitive to inhibition by HSV; HSV infection of NK cells by cell-to-cell spread or direct infection renders NK cells unable to lyse normally sensitive target cells (York and Johnson, personal communication). Recently, human herpesvirus 6 (HHV-6) has been shown to directly infect and kill NK cell clones (25). Thus, the infection of cytotoxic effector cells may be a general mechanism by which herpesviruses avoid surveillance from cells of the innate and adaptive immune systems.

Although infection of CTL with HSV-1 inhibits their lytic function, the mechanism of this inhibition is not known. A host protein shut-off by vhs is not involved in the inhibition of CTL-mediated lysis because viruses mutant in vhs inhibit CTL lytic function comparable to wild-type HSV-1. It is conceivable that the expression of a cellular protein(s) induced upon infection or a viral protein(s) may be involved in inhibiting any stage of the CTL lytic cycle including adhesion and specific recognition, signal transduction or release of cytotoxic effector molecules.

These findings provide evidence that HSV is involved in immunosuppression of cell-mediated immunity. The success of HSV in the human population may be a result of its ability to evade immune recognition and through direct suppression of mechanisms involved in its control. The inactivation of CTL, monocytes and NK cells by HSV-FB may occur within herpetic lesions preventing the control of infection and permitting the spread of virus. Further, our results suggest that any CTL would be sensitive to the inhibitory effects of HSV which may render an

individual with active HSV infections susceptible to other viral infections controlled by CTL.

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Table I. Titre of HSV-1 produced by HSV-FB in a sandwich assay[a]

Time of HSV-1 infection (h)	Before effectors added		Before targets added	
	PFU/well[b]	PFU/CTL[c]	PFU/well	PFU/CTL
2	1500	.003	700	.001
20	$4x10^6$	8	$5x10^6$	10

[a]FB were infected for .5 or 18.5 hours with 10 PFU HSV-1, trypsinized and $1x10^4$ cells/well were added to a 96-well plate. Following adherance for 1.5 hours, supernatant was removed corresponding to the time immediately prior to the addition of effectors in a sandwich assay (Before effectors added) or immediately prior to the addition of ^{51}Cr-labeled target cells in a sandwich assay (Before targets added).

[b]HSV-1 titre in the supernatant from 8 wells was measured and PFU/well was calculated.

[c]PFU/CTL was determined by dividing the PFU/well by $5x10^5$ CTL/well which represents an effector:target ratio of 50:1, the typical number of CTL added in a sandwich assay of bulk allo-CTL.

Figure 1. Inhibition of allo-CTL mediated lysis by HSV-FB does not involve vhs function. Allo-CTL were incubated for 3 hours in a sandwich assay with uninfected FB (un) or FB infected for 2 or 20 hours with HSV-1F, PAA^R5 (PAAR), vhsA or vhsB. Following incubation, ^{51}Cr-labeled allo-LCL were added for 5 hours in a ^{51}Cr-release assay. Results shown are with an E:T ratio of 50:1; error bars represent the standard deviation from the mean of triplicate wells.

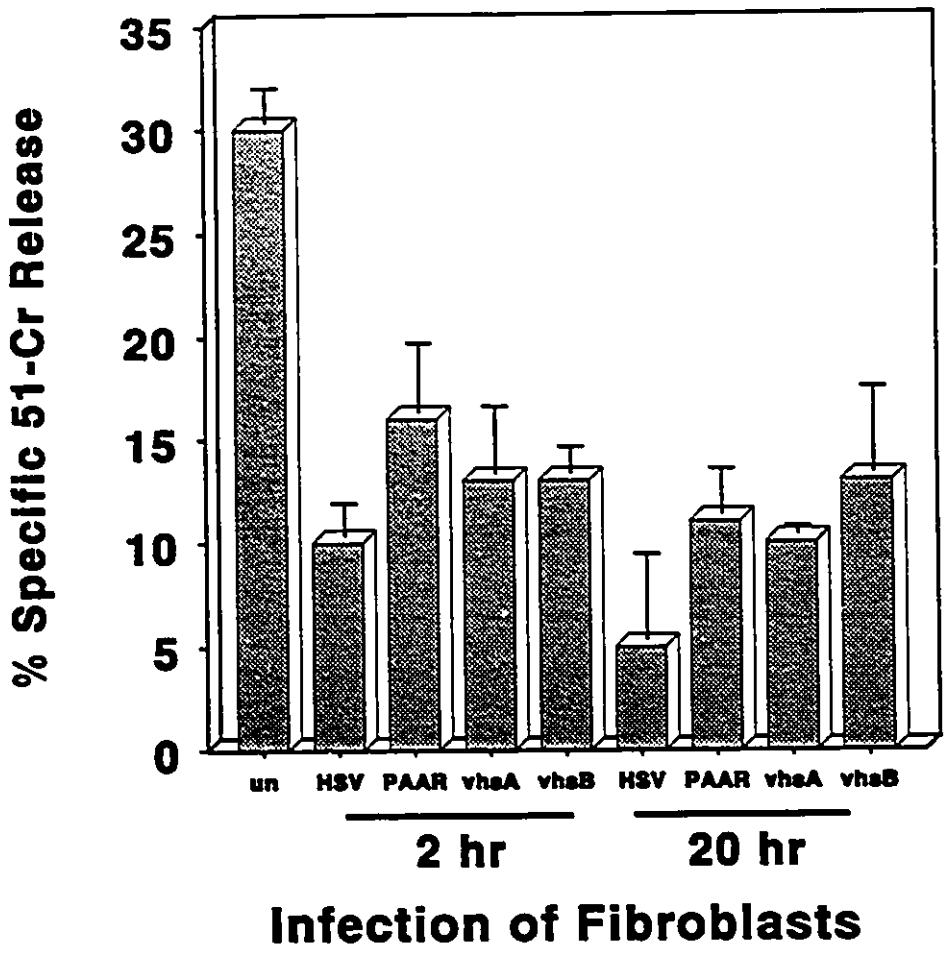
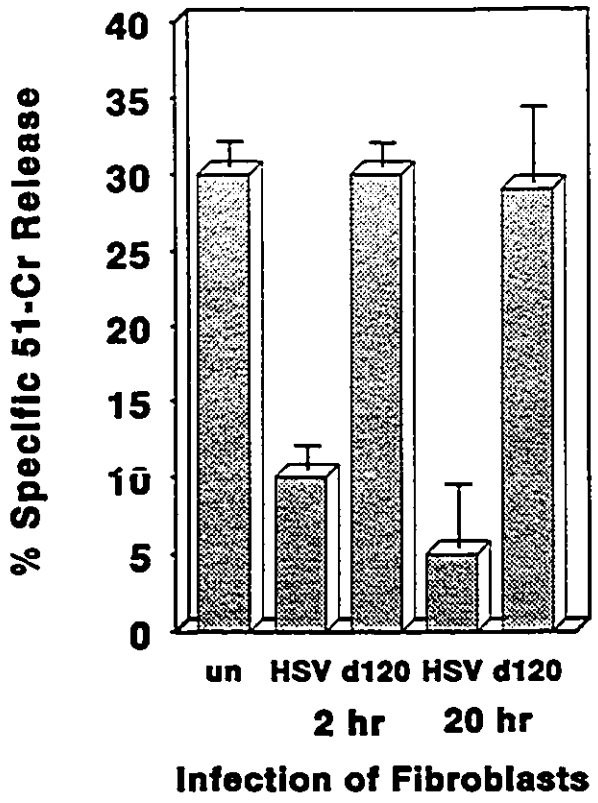

% Specific 51-Cr Release
35
30
25
20
15
10
5
0
un
HSV
PAAR
vhsA
vhsB
HSV
PAAR
vhsA
vhsB
2 hr
20 hr
Infection of Fibroblasts

Figure 2. The expression of ICP4 in HSV-FB is required to inhibit CTL lytic function. Allo-CTL (A) or 3G5 cells (B) were incubated for 3 hours in a sandwich assay with uninfected FB (un) or FB infected for 2 or 20 hours with 10 PFU/cell HSV-1 or d120 prior to the addition of ^{51}Cr-labeled target cells (allo LCL for allo-CTL; A2+ LCL for 3G5 cells). Results shown are with an E:T ratio of 50:1 in (A) or 0.5:1 in (B); error bars represent the standard deviation from the mean of triplicate wells.

A


% Specific 51-Cr Release
40
35
30
25
20
15
10
5
0
un
HSV d120
HSV d120
2 hr
20 hr
Infection of Fibroblasts

B

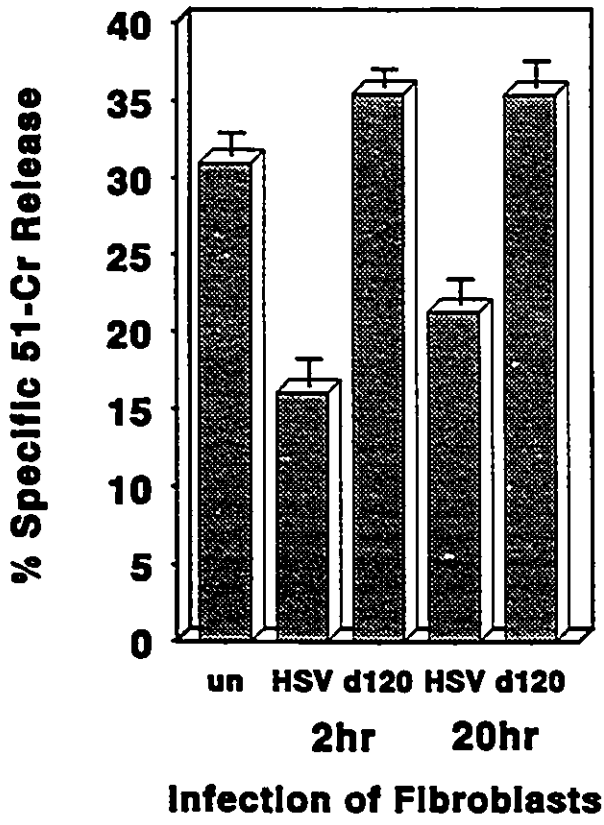

% Specific 51-Cr Release
40
35
30
25
20
15
10
5
0
un
HSV d120
HSV d120
2hr
20hr
Infection of Fibroblasts

Figure 3. Role of HSV-specific glycoproteins in inhibition of allo-CTL mediated lysis by FB infected with HSV-1 for 20 hours. Allo-CTL were incubated for 3 hours in a sandwich assay with uninfected FB (un), FB infected for 20 hours with 10 PFU/cell HSV-1F (1F), HSV-2 (333), or viruses mutant in glycoproteins B, C, D, E, H, I, K or L. Following incubation, ^{51}Cr-labeled allo-LCL were added for 5 hours in a ^{51}Cr-release assay. Results shown are with an E:T ratio of 50:1; error bars represent the standard deviation from the mean of triplicate wells. *inhibition of allo-CTL lysis compared with lysis by allo-CTL incubated with uninfected FB ($p<0.05$).

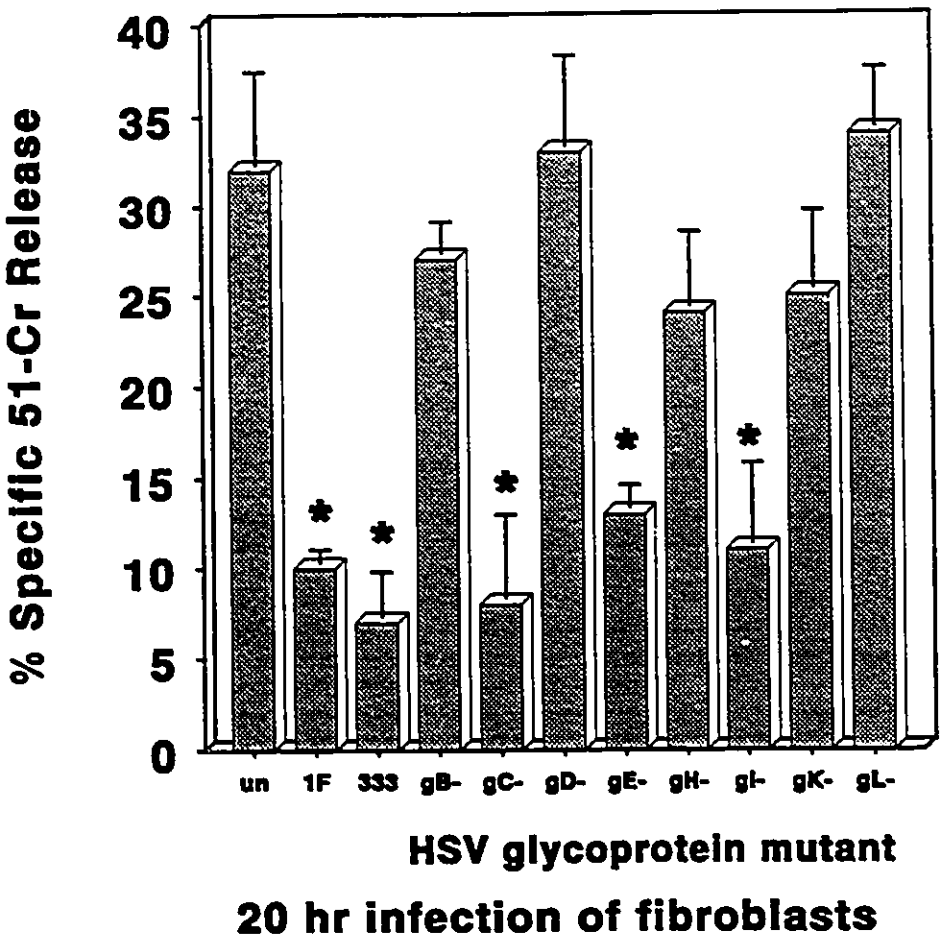

% Specific 51-Cr Release
40
35
30
25
20
15
10
5
0
un
1F
333
gB-
gC-
gD-
gE-
gH-
gI-
gK-
gL-
HSV glycoprotein mutant
20 hr infection of fibroblasts

Figure 4. The production of infectious virus from HSV-FB is not required to inhibit allo-CTL mediated lysis by FB infected with HSV-1 for 2 hours. Allo-CTL were incubated for 3 hours in a sandwich assay with uninfected FB (un), FB infected for 2 hours with 10 PFU/cell HSV-1F (1F), or a viruses mutant in glycoproteins D (gD-) or L (gL-). Following incubation, ^{51}Cr-labeled allo-LCL were added for 5 hours in a ^{51}Cr-release assay. Results shown are with an E:T ratio of 50:1; error bars represent the standard deviation from the mean of triplicate wells. *inhibition of allo-CTL lysis compared with lysis by allo-CTL incubated with uninfected FB ($p<0.05$).

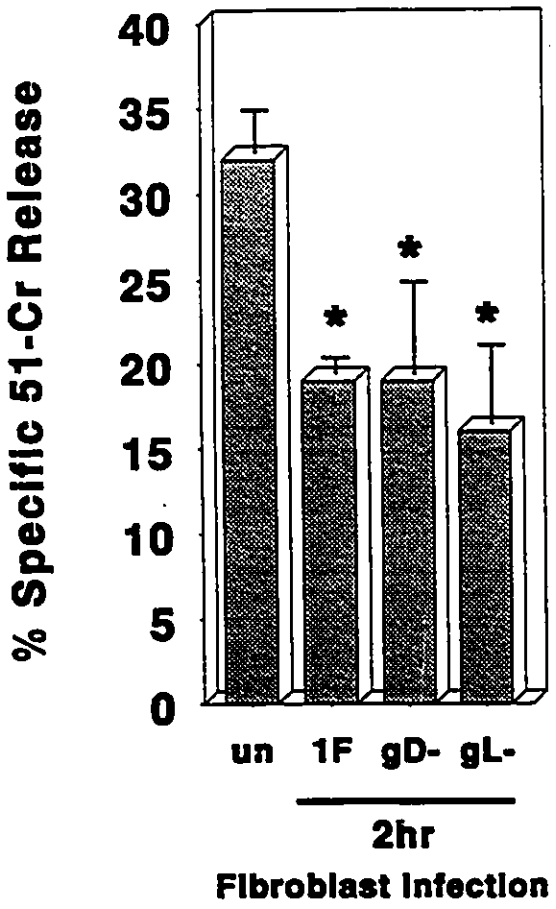

% Specific 51-Cr Release
40
35
30
25
20
15
10
5
0
*
*
*
un
1F
gD-
gL-
2hr
Fibroblast Infection

Figure 5. Expression of HSV-specific proteins in CTL exposed to HSV-FB. 3G5 cells were incubated for 3 hours with uninfected FB or FB infected with HSV-1 for 2 or 20 hours. 3G5 were removed from the FB (removed) and the remaining 3G5 cells were trypsinized (trypsinized). Both fractions were layered onto Percoll gradients as described in Materials and Methods. Isolated 3G5 cells and HSV-infected Vero cells were metabolically labeled with ^{35}S-methionine and cell lysates prepared. ICP4 (α4) and gB were immunoprecipitated with protein A-Sepharose and 58S or 15βB2, respectively. The immature form of glycoprotein B is indicated by pgB.

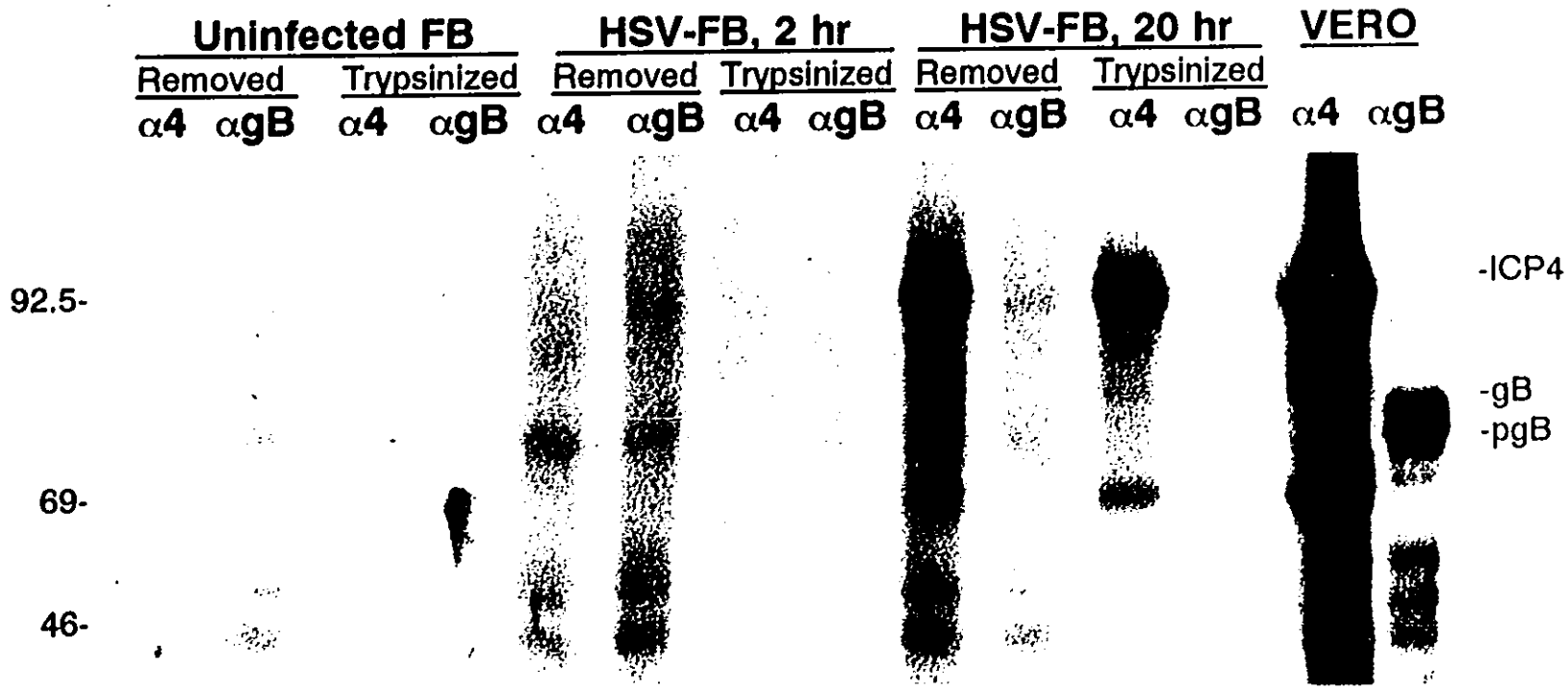

Uninfected FB
HSV-FB, 2 hr
HSV-FB, 20 hr
VERO
Removed
Trypsinized
Removed
Trypsinized
Removed
Trypsinized
α4 αgB α4 αgB α4 αgB α4 αgB α4 αgB α4 αgB α4 αgB
92.5-
69-
46-
-ICP4
-gB
-pgB

Figure 6. Inhibition of allo-CTL lytic activity by direct infection with HSV-1. Allo-CTL were infected for 2 hours with various amounts of HSV-1 prior to use in a ^{51}Cr-release assay with allo LCL or auto LCL as target cells. Results shown are with an E:T ratio of 50:1; error bars represent the standard deviation from the mean of triplicate wells. *inhibition of allo-CTL compared with lysis by uninfected allo-CTL ($p<0.05$).

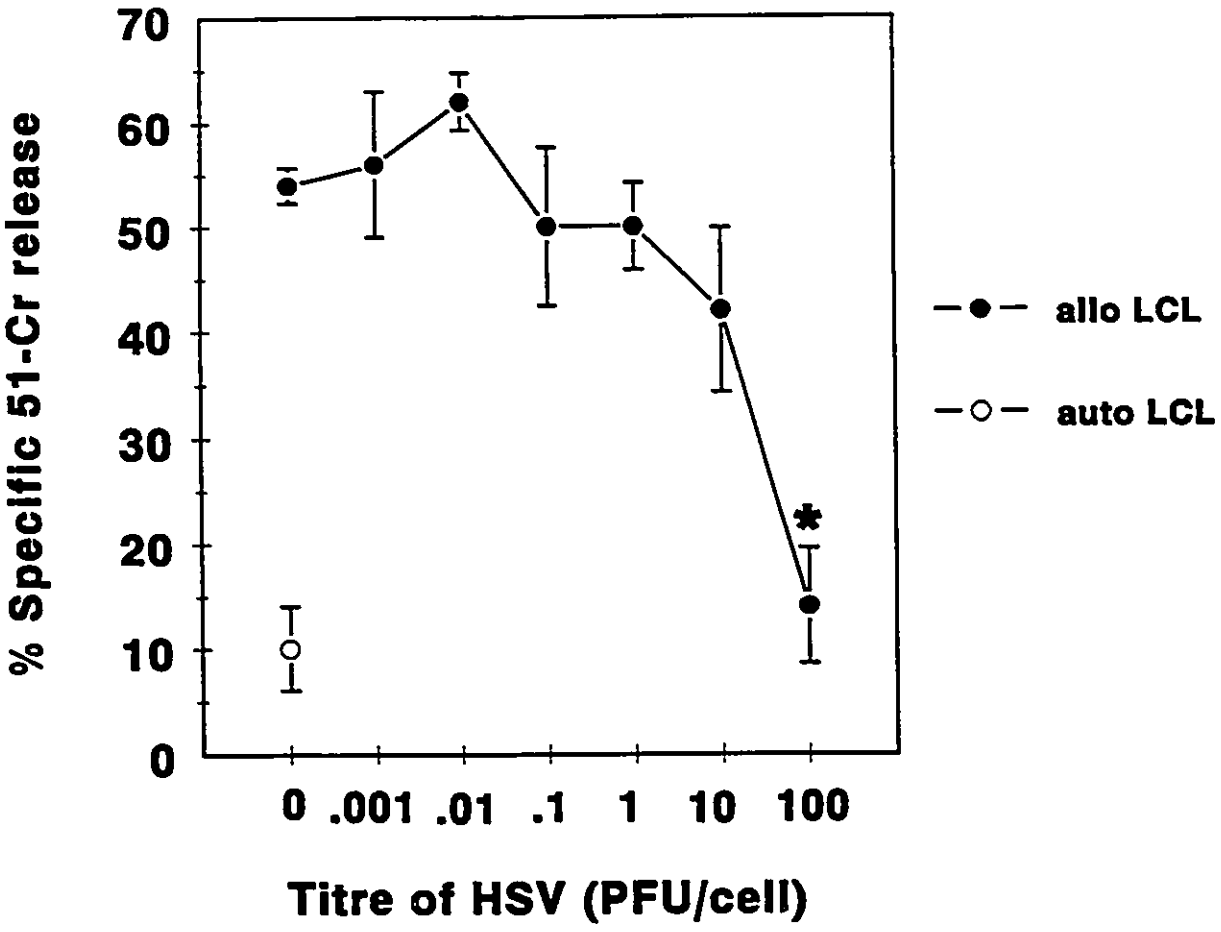
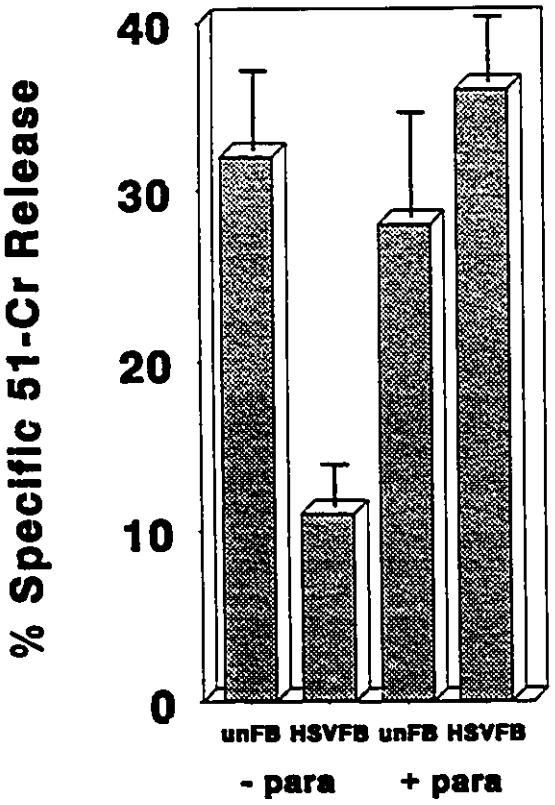

70
60
50
40
30
20
10
0
% Specific 51-Cr release
0 .001 .01 .1 1 10 100
Titre of HSV (PFU/cell)
allo LCL
auto LCL
*

Figure 7. FB infected with HSV-1 for 20 hours do not inhibit allo-CTL lytic activity when fixed with paraformaldehyde. Allo-CTL were incubated for 3 hours in a sandwich assay with uninfected FB (unFB) or FB infected with HSV-1 for 20 hours previously treated with (+para) or without (-para) 1% paraformaldehyde. Following incubation, ^{51}Cr-labeled allo-LCL were added for 5 hours in a ^{51}Cr-release assay. Results shown are with an E:T ratio of 50:1; error bars represent the standard deviation from the mean of triplicate wells.


% Specific 51-Cr Release
40
30
20
10
0
unFB HSVFB unFB HSVFB
- para
+ para

CHAPTER 6

DISCUSSION

A. Summary of Results.

The first aspect of human CTL responses to HSV studied involved the specificity of human anti-HSV CTL generated from a single in vitro exposure to HSV. The approach taken was to utilize recombinant adenovirus vectors encoding gB (AdgB) or gD (AdgD) of HSV-1. Recombinant virus-infected autologous LCL from HSV-seropositive donors were the target cells used. Of six HSV-seropositive donors tested, anti-HSV CTL from one individual lysed LCL infected with HSV-1, and recombinant vaccinia virus vectors encoding gB (vgB11) or gD (vgD52). gB- and gD-specific CTL were $CD4^+$ and $CD8^+$ T cells. Anti-HSV CTL from the remaining 5 donors recognized HSV-infected LCL only. Although AdgB- and AdgD-infected LCL expressed gB and gD as detected by immunoprecipitation, less than 2% of cells were positive for glycoprotein expression as detected by FACS analysis. The low expression of inserted gene products in recombinant adenovirus-infected LCL was confirmed using a recombinant adenovirus encoding the β-galactosidase gene (Ad5LacZ); 0.01% of Ad5LacZ-infected LCL expressed detectable β-galactosidase activity. Therefore, LCL infected with recombinant adenovirus vectors were not recognized by anti-HSV CTL due to low levels of inserted gene product expression and restricted infection of LCL.

Because LCL were poor targets for adenovirus replication and expression of inserted gene product, human fibroblasts (FB) were chosen as potential target cells for anti-HSV CTL since they are permissive to adenovirus infection.

However, when HSV-infected FB (HSV-FB) were tested as targets for anti-HSV CTL, no specific lysis was detected. HSV-FB were not only resistant to lysis by anti-HSV CTL but also to allo-antigen specific CTL (allo-CTL). HSV-FB were not resistant to all forms of cell-mediated cytotoxicity since HSV-FB were lysed by cells mediating antibody-dependent cellular cytotoxicity (ADCC).

The exposure of anti-HSV CTL or allo-CTL to HSV-FB rendered the CTL unable to lyse normally sensitive target cells. Infection of FB with HSV for as little as 2 hours profoundly inhibited the lytic activity of human CTL. This inhibitory phenomenon appeared to be HSV-specific because exposure of CTL to FB infected with vaccinia virus or adenovirus had no effect on CTL lytic activity. Cell-to-cell contact was necessary for the inhibition of CTL lytic function and a soluble factor produced from HSV-FB was not required.

To study the mechanism of inhibition of CTL lytic function by HSV-FB, a number of mutant viruses were employed. Virus-induced shut-off of host protein synthesis was not involved in the inhibition of CTL activity. However, the expression of ICP4, an immediate-early protein of HSV, was necessary. The expression of HSV glycoprotein genes essential for viral infectivity in vitro were required for FB infected with HSV-1 for 20 hours, but not 2 hours, to inhibit CTL-mediated lysis. Further, FB infected with HSV-1 for 20 hours were infected as measured by viral protein expression. Cell-to-cell spread of HSV appeared to be the primary mode of viral transmission from FB infected for 20 hours to CTL since (1) supernatant from HSV-FB did not inhibit CTL lytic function and (2)

paraformaldehyde-fixed cells also did not inhibit CTL-mediated lysis. In contrast, FB infected with HSV for 2 hours did not express viral proteins and production of infectious virus was not required for HSV-FB to inhibit CTL lytic function. In summary, two distinct mechanisms of inhibition of CTL lytic function by HSV-FB were discovered: one that occurred early in the HSV replication cycle requiring ICP4 but not infectious virus or virus-induced host shut-off, and one that occurred late and involving the infection of CTL by cell-to-cell transmission of HSV from infected FB.

B. Phenotype of anti-HSV CTL: In vivo relevance of $CD4^+$ CTL.

Many studies have focussed on the phenotype of human anti-HSV CTL generated in vitro. The source of CTL has been the peripheral blood of HSV-seropositive individuals that was stimulated by exposure to HSV antigen (Schmid, 1988; Yasukawa et al., 1983; Yasukawa and Zarling, 1984a; Torpey et al., 1989), UV-HSV-absorbed fibroblasts (Yasukawa et al., 1989) and PHA-activated HSV-infected PBMC (Tigges et al., 1992; Maccario et al., 1993). In general, the phenotype of anti-HSV CTL has depended on the mode of stimulation; $CD4^+$ cells predominate when PBMC were stimulated with HSV antigen while $CD8^+$ cells predominate when PBMC were stimulated with cell-associated HSV. These results lend support to the current dogma of antigen presentation. Peptide fragments from endogenously synthesized antigens associate with MHC class I molecules and are presented to $CD8^+$ T cells (Brodsky and Guagliardi, 1991). In contrast, class II molecules can present peptides from exogenously or endogenously processed

antigens to $CD4^+$ T cells (Brodsky and Guagliardi, 1991).

In the present study, PBMC stimulated with live HSV generated $CD4^+$ and $CD8^+$ anti-HSV CTL. These findings suggest that both pathways of antigen presentation are employed by antigen presenting cells upon exposure to HSV. Free virus is processed by antigen presenting cells from PBMC (monocytes, B lymphocytes) by the endocytic route and peptides presented by class II molecules would stimulate and expand memory $CD4^+$ anti-HSV CTL. In contrast, virus is able to infect cells and express viral proteins which can be processed and presented by class I molecules to expand memory $CD8^+$ anti-HSV CTL or by class II molecules to expand memory $CD4^+$ anti-HSV CTL. It is expected that both forms of antigen presentation would occur in an HSV lesion since free HSV and HSV-infected cells would be present. The high frequencies of $CD4^+$ and $CD8^+$ anti-HSV CTL from the peripheral blood indicate that this may be the case (Schmid, 1988; Yasukawa et al., 1989).

The in vivo relevance of $CD4^+$ anti-HSV CTL has been controversial. $CD4^+$ CTL have been described for a number of viral infections including rabies virus, measles virus, influenza virus, VZV, CMV and HSV (reviewed in Schmid and Mawle, 1991). The major objection against a direct combat role for $CD4^+$ T cells is that few cell types in the body express MHC class II molecules on the cell surface, the recognition structure for $CD4^+$ T cells (Schmid and Mawle, 1991). However, interferon-γ, produced upon viral infections including HSV (Cunningham et al., 1985), induces the expression of class II molecules on a number of cell

types (Young and Pepose, 1987). Thus, the in vivo induction of MHC class II expression within a herpetic lesion by interferon-γ would allow the stimulation and expansion of $CD4^+$ HSV-specific CTL.

The most compelling evidence for an in vivo role for $CD4^+$ T cells in HSV infections comes from the characterization of cells within an active HSV infection. $CD4^+$ T cells, Langerhans cells, dendritic macrophages and other phagocytic cells are the only immune cells found inside a lesion during the first several days (Cunningham et al., 1985). $CD8^+$ T cells and NK cells do not infiltrate until day 3 or 4 when lesions have begun to resolve. Further, keratinocytes within or adjacent to herpetic lesions uniformly express class II molecules on their surface (Cunningham et al., 1985). Recently, HSV-specific CTL, cloned from herpetic lesions, were exclusively $CD4^+$ (Koelle and Corey, personal communication). Although the function of $CD4^+$ T cells within a lesion is not known, their existence at early stages in disease suggests an important role of these cells in disease resolution. The normally ascribed helper functions of $CD4^+$ T cells, such as cytokine release, may be augmented by direct cytotoxicity against HSV-infected class II-expressing cells (Schmid and Rouse, 1992).

Characterization of the phenotype of CTL is important to determine the MHC restricting elements involved in control of viral infections and to determine what constitutes "relevant immunity". For example, if $CD4^+$ or $CD8^+$ T cells are involved in control of infections, therapeutic or preventative strategies would be directed at the generation of the appropriate subpopulation. Live vaccine preparations may

expand $CD8^+$ anti-HSV CTL while inactivated vaccines may be useful to expand $CD4^+$ CTL. In support of this, humans immunized with an HSV-2 subunit vaccine successfully generated $CD4^+$ HSV-specific CTL (Zarling et al., 1988), although the presence of $CD8^+$ HSV-specific CTL or the protective effect of vaccination was not determined. Since no animal model appropriately reflects human HSV infections, determination of relevant immunity in humans will be difficult.

C. Specificity of human CTL specific for herpesviruses

HSV glycoproteins and immediate-early proteins have been shown to act as target antigens for murine anti-HSV CTL (Witmer et al., 1990; Hanke et al. 1991; Martin and Rouse, 1992). In human studies, gB and gD could act as target antigens for anti-HSV CTL clones in the individuals tested (Zarling et al., 1986a; Tigges et al., 1992). Repeated rounds of stimulation of PBMC with HSV antigen, HSV glycoproteins, or HSV-infected cells was required to generate CTL clones (Zarling, 1986a; Tigges et al., 1992). This process can expand low frequencies of CTL specific for individual proteins from the peripheral blood. However, it does not provide an accurate assessment of antigen specificity from freshly isolated blood. Thus, this study was undertaken to determine if PBMC stimulated with a single *in vitro* exposure to HSV could generate CTL specific for gB or gD. Freshly isolated PBMC have non-specific NK cell cytotoxicity but no HSV-specific CTL activity, and thus, *in vitro* antigen stimulation was necessary. The HSV-specific memory T cell populations would be expanded, and not naive T cells, since anti-HSV CTL are not generated in HSV-seronegative individuals (Sethi et al., 1980).

LCL infected with recombinant vaccinia viruses encoding gB and gD were chosen as the target cells because they can be lysed by human anti-HSV CTL clones (Zarling et al., 1986b; Tigges et al., 1992). Of six HSV-seropositive donors tested, only one donor had detectable gB- and gD-specific CTL activity from bulk cultures of anti-HSV CTL. Depletion studies revealed that gB- and gD-specific CTL were composed of $CD4^+$ and $CD8^+$ CTL. Anti-HSV CTL from the remaining five donors lysed HSV-infected LCL only. Therefore, although gB and gD can serve as target antigens for bulk cultures of human anti-HSV CTL, the majority of the bulk CTL response in most patients tested was directed at HSV antigens other than gB and gD.

It is not clear from earlier studies of $CD4^+$ anti-HSV CTL clones whether gB- and gD-specific clones could be generated from any HSV-seropositive person. gB- and gD-specific $CD4^+$ CTL clones were isolated from only two HSV-seropositive people (Zarling et al., 1986a). A gD-specific $CD8^+$ CTL clone was isolated from a single HSV-2 seropositive donor and one donor tested had detectable gD-specific CTL from bulk cutures of anti-HSV CTL (Tigges et al., 1992). Therefore, the prevalence of CTL specific for gB and gD is not known. Although only 6 donors were tested, the present results suggest that gB- and gD-specific CTL are not demonstrable from the majority of bulk cultures of anti-HSV CTL. Although gB- and gD-specific CTL in these donors may be detected by CTL cloning, it is clear that other HSV proteins serve as target antigens for human anti-HSV CTL. In support of this, Tigges et al. (1992) have shown that $CD8^+$ anti-HSV CTL clones

are specific for diverse virion proteins including structural and non-structural proteins.

The specificity of human CTL to other herpesvirus infections varies from multiple specificities of anti-VZV CTL and anti-EBV-CTL to a relatively restricted specificity of anti-CMV CTL. VZV proteins gpI, gpIV (HSV gI homologue) and IE62 (HSV ICP4 homologue) can serve as target antigens for $CD4^+$ or $CD8^+$ CTL, although these were not major target antigens (Arvin et al., 1991; Huang et al., 1992). Of sixteen EBV-immune donors tested, anti-EBV CTL were specific for multiple EBV latent proteins normally expressed in LCL (Murray et al., 1992). However, EBNA 3 proteins were recognized most frequently and no anti-EBV CTL were specific for EBNA 1, EBNA LP or LMP (Murray et al., 1992). In contrast to VZV and EBV, the anti-CMV CTL response in the donors tested was primarily directed at a single CMV immediate-early protein, p72, although a minor population recognized CMV-gB (Borysiewicz et al., 1988a). No other CMV proteins have been shown to serve as target antigens for anti-CMV CTL.

It is apparent that the specificity of human anti-HSV CTL is largely unknown. It is likely that multiple HSV proteins will serve as target antigens for human anti-HSV CTL depending on an individual's HLA type. To produce an effective vaccine, both the phenotype and specificity of T cells conferring protective immunity needs to be determined.

D. Recombinant adenovirus vectors in the study of human anti-HSV CTL

Although recombinant vaccinia viruses encoding individual HSV genes have

been widely used in the murine and human systems, research employing recombinant adenovirus vectors has been restricted to the murine system. Immunization of mice with recombinant adenoviruses encoding gB of HSV (AdgB) have protected mice from subsequent lethal challenge with HSV-2 (McDermott et al., 1989; Gallichan et al., in press). In vitro studies indicated that anti-HSV CTL generated from H-2^b or H-2^d mice lysed targets infected with AdgB but not AdgC or AdgD (Witmer et al., 1990; Johnson, 1991). Recombinant adenoviruses were also used to map an immunodominant epitope within gB that was recognized by H-2^b anti-HSV CTL (Hanke et al., 1991). With the successful use of recombinant adenoviruses in the murine system, it was anticipated that these vectors would be useful in assessing the specificity of human anti-HSV CTL.

AdgB- and AdgD-infected LCL expressed gB and gD, respectively, as determined by immunoprecipitation. However, these cells were not lysed by anti-HSV CTL that lysed LCL infected with recombinant vaccinia viruses encoding gB or gD. The inability of LCL infected with recombinant adenoviruses to serve as targets for anti-HSV CTL was due to the low percentage of cells expressing the inserted gene product. FACS analysis revealed that the expression of gB or gD from LCL infected with recombinant adenoviruses was no different than from LCL infected with control adenoviruses. Further, LCL infected with a recombinant adenovirus encoding the LacZ gene (Ad5LacZ) expressed β-galactosidase activity in 0.01% of LCL. Therefore, the expression of insert genes in recombinant adenoviruses, normally expressed in permissive cell lines, is greatly reduced in

LCL.

Adenovirus was originally isolated from adenoids and tonsils of pateints with acute respiratory diseases (Enders et al., 1956). Although lymphoid cells compose 75% of adenoid tissue, isolation of adenovirus from lymphocytes within adenoid tissue was unsuccessful (Strohl and Schlesinger, 1965). Peripheral blood lymphocytes were non-permissive for adenovirus infection, and PHA-activation of PBL enhanced viral replication (Horvath and Weber, 1988). However, Lavery et al. (1987) demonstrated that with one exception, established human B and T cell lines supported adenovirus replication, RNA synthesis, and production of infectious virus. Further, LCL were found to contain Group C (Ad1, Ad2, Ad5 or Ad6) adenovirus DNA and infectious virus could be isolated (Horvath et al., 1986).

In contrast to these studies, the results of the present study suggest that although insert gene products can be detected in a very small percentage of LCL infected with recombinant adenoviruses, the expression of insert gene products in the majority of cells is restricted. Although the replication of recombinant adenoviruses in LCL may be different than the replication of wild-type adenoviruses, this does not appear to be the case for other cells types where recombinant adenovirus infection is as efficient as wild-type viruses at least in virus production (Johnson, 1991). However, the present study does not address this issue. Only the expression of insert genes, and not adenoviral genes, were measured. The integration of foreign genes into the non-essential E3 region should have no effect on the replication of adenovirus in permissive cell types.

The recombinant adenoviruses used contain an upstream SV40 promoter although this promoter does not initiate transcription (Johnson, 1991). More recently, recombinant adenoviruses have been constructed to contain an open reading frame inserted into the E3 region so that no promoter is adjacent to the foreign gene (Johnson, 1991). The expression of foreign genes in these vectors appear to be better, although expression in LCL has not been examined.

Human dermal FB are easily obtained by skin biopsy and were found to be permissive for recombinant adenovirus replication and expression of insert genes (Posavad, unpublished observations). Therefore, fibroblasts infected with recombinant adenoviruses could be used as potential target cells for human anti-HSV CTL. However, as discussed in a later section, a positive control for this system is not available. Human FB (Chapter 4; Koelle et al., 1993) or keratinocytes (Koelle et al., 1993) infected with HSV are resistant to lysis by human anti-HSV CTL. A human cell type that is permissive for adenovirus replication, is easily obtained from HSV-seropositive donors, and that is sensitive to lysis by CTL will be required to study the potential of recombinant adenoviruses to serve as human anti-HSV CTL targets.

Although recombinant adenoviruses encoding individual HSV proteins were not effective in characterizing the specificity of human anti-HSV CTL when LCL were used as target cells, further research into the utility of these vectors as human vaccines is warranted. U.S. military recruits have been successfully protected from upper respiratory infections with adenoviruses type 4 and 7 with no

adverse reactions (Meiklejohn, 1983). The relative stability of adenoviruses, the ease to which they can be produced and the current success in protecting mice from lethal challenge make them promising vaccine candidates for preventation of HSV infection. Further, since adenoviruses are mucosal pathogens, they may make effective vaccines against sexually-transmitted diseases such as HSV and HIV which spread mucosally.

E. HSV-infected fibroblasts as targets for human anti-HSV CTL

To further characterize the specificity of human anti-HSV CTL using recombinant adenoviruses, human dermal fibroblasts were chosen as target cells. Skin fibroblasts can be obtained by skin biopsy from the forearm providing autologous target cells. AdgB- and AdgD-infected FB express abundant levels of insert gene product in the majority of cells as detected by immunoprecipitation and immunofluorescence (Posavad, unpublished data).

When FB were used as target cells for human anti-HSV CTL, HSV-infected FB (HSV-FB) were not lysed by $CD4^+$ or $CD8^+$ HSV-specific CTL. This experiment was repeated at least 10 times with anti-HSV CTL from different HSV-seropositive donors and at no time post-infection were HSV-FB lysed by these CTL. These results were surprising since others had shown that HSV-FB can be killed by human anti-HSV CTL (Yasukawa et al., 1989; Sethi, 1980). Further, human CTL specific for CMV, also a member of the herpesviruses family, lysed virally-infected FB (Borysiewicz et al., 1988b). Due to these observations, the focus of this thesis was directed at the mechanism of resistance of HSV-FB to human anti-HSV CTL.

HSV-FB were also resistant to human allo-antigen specific CTL (allo-CTL) in a time-dependent manner; the longer FB were infected with HSV, the lower the killing by allo-CTL. In contrast, HSV-FB were sensitive to lysis by antibody-dependent cellular cytotoxicity using freshly isolated PBMC and anti-HSV antibodies. The resistance of HSV-FB to CTL was confirmed in another study that demonstrated that HSV infection of human FB or keratinocytes inhibited the recognition by $CD8^+$ CTL clones (Koelle et al., 1993). The resistance of HSV-FB to lysis involved a viral late gene product and possibly the reduction of of HLA class I antigen surface expression (Koelle et al., 1993). HSV-FB were also shown to be resistant to NK and LAK cell-mediated lysis (Confer et al., 1990)

F. Immunosuppression of CTL by HSV-FB

Confer et al. (1990) determined that the exposure of NK or LAK cells to HSV-FB inhibited their ability to lyse normally sensitive target cells. In order to determine if a similar phenomenon occurred to inhibit CTL lytic function, anti-HSV CTL or allo-CTL were incubated with HSV-FB for various times. Subsequently, ^{51}Cr-labeled target cells, normally sensitive to killing by CTL, were added for 5 hours in a ^{51}Cr-release assay. These "sandwich assays", as coined by Confer et al. (1990), allow one to observe the effect of HSV-FB on the lytic function of CTL. Anti-HSV CTL or allo-CTL exposed to FB infected for 2, 4 or 20 hours with HSV were inhibited in lysing their normally sensitive target cells. The degree of inhibition of CTL lytic function was directly proportional to the length of time the CTL were exposed to the HSV-FB. This inhibitory phenomenon was virus-specific

as FB infected with adenovirus or vaccinia virus did not inhibit CTL lytic activity.

Soluble factors produced by different cell types have been shown to inhibit the proliferative or lytic phases of human CTL. Transforming growth factor β, produced from an HSV-2-induced murine tumor cell, suppressed proliferative responses in a mixed lymphocyte reaction from PBMC (Prabhu Das et al., 1991). A molecule released from $CD8^{+}CD57^{+}$ T cells obtained from AIDS patients inhibited the lytic ability of MHC-restricted CTL (Sadat-Sowdi et al., 1991). BCRF1, an EBV protein homologous to IL-10, may inhibit CTL lytic function similar to IL-10 (Bejarano et al., 1992). In the present study, the inhibition of CTL lytic activity by HSV-FB did not involve a soluble factor produced from HSV-FB since supernatant from HSV-FB did not inhibit lytic function. Further, when CTL were separated from HSV-FB by a porous membrane that allowed soluble factors, but not cells, to pass, full lytic function was retained. Therefore, cell-to-cell contact between HSV-FB and CTL was required for HSV-FB to inhibit CTL lytic activity.

Two distinct mechanisms of inhibition of CTL lytic function by HSV-FB appeared to be operative. The first mechanism occurred early in the HSV replication cycle (2 hours post-infection) and involved the expression of ICP4 but not the production of infectious virus or virus-induced shut-off of host protein synthesis. Further, CTL exposed to FB infected with HSV-1 for 2 hours did not express detectable levels of viral proteins indicating that demonstrable infection of CTL with HSV was not necessary for inhibition. In contrast, CTL exposed to FB infected with HSV-1 for 20 hours expressed HSV-specific proteins indicating that

they were infected with HSV-1. The expression of glycoproteins essential for viral infectivity was required confirming that infection of CTL with HSV was the mechanism of inhibition of CTL lytic. Cell-to-cell spread of HSV was the likely mode of viral transmission since supernatant from FB infected for 20 hours or paraformaldehyde-fixed cells did not inhibit CTL-mediated lysis. Although direct infection of CTL by HSV inhibits their lytic ability, levels of cell-free virus in the supernatant of HSV-FB were too low to significantly inhibit CTL lytic activity.

Cell-to-cell transmission of virus from infected cells to neighbouring uninfected cells has been documented for a number of viruses. Cell-to-cell transmission of virus occurs by the formation of intercellular bridges or channels that allow the movement of virus (Zsak et al., 1993). Vaccinia virus, an enveloped, double-stranded DNA poxvirus, spreads by direct infection with extracellular enveloped virus or by cell-to-cell transmission with cell-associated enveloped virus (Blasco and Moss, 1992). HIV, an enveloped retrovirus, can spread by cell-to-cell transmission (Sato et al., 1992). HIV-infected cells, expressing gp120/gp41 proteins of HIV, can fuse with uninfected cells by interaction with CD4 molecules on their surface (Sato et al., 1992). Pseudorabies virus (PrV), an alpha herpesvirus that infects pigs, requires the expression of gI (HSV gE homologue) (Zsak et al., 1992), gII (HSV gB homologue) (Heffner et al., 1993) and gH (HSV gH homologue) (Peeters et al., 1992) for cell-to-cell transmission. PrV mutants that are unable to spread by cell-to-cell transmission are avirulent demonstrating that direct cell-to-cell transmission is the pathogenically important mode of PrV

spread (Heffner et al., 1993).

Cell-to-cell transmission of HSV has also been reported (Navarro et al., 1992). Complement-independent neutralizing antibodies to gB, gD, or gH, which prevent viral penetration into cells but not virus attachment, inhibited plaque formation (Buckmaster et al., 1984; Highlander et al., 1988; Minson et al., 1986; Navarro et al., 1992). The authors concluded that the spread of infection to uninfected cells did not depend on the release of virus but rather on the expression of HSV-1 glycoproteins on the cell membrane of infected cells (Buckmaster et al., 1984; Highlander et al., 1988; Minson et al., 1986; Navarro et al., 1992). Due to the high levels of virus required to inhibit CTL lytic activity and the relatively low levels of cell-free virus present in the supernatant of FB infected for 20 hours as well as the requirement for the expression of gB, gD and gH (as well as gL), cell-to-cell spread of HSV, from infected FB, to CTL was the mode of viral transmission. The cell-to-cell spread of HSV-1 may be a quicker and more efficient way for viruses to spread since mature virion production is not required. The cell-to-cell transmission of HIV occurred within minutes and did not require the budding or mature virus production (Sato et al.,1992).

Direct HSV infection of cells of the innate immune system leads to their inactivation. Direct infection or cell-to-cell spread of virus from infected FB to lymphokine-activated killer (LAK) cells inhibits LAK cell function (York and Johnson, personal communication). Similarly, HHV-6 can infect human NK cell clones leading to their destruction (Lusso et al., 1993). The present study extends

these findings and demonstrates that innate and adaptive immune systems are sensitive to the immunosuppressive ability of HSV.

The mechanism of inhibition of CTL lytic function by direct infection or cell-to-cell transmission of HSV-1 is not known. A host protein shut-off by vhs is not involved in the inhibition because vhs mutants inhibited CTL lytic function as well as wild-type HSV. However, not all host proteins are shut-off by HSV; the synthesis of some host proteins can be induced upon infection with HSV (Sarmiento and Kleinerman, 1990). Alternatively, a viral protein may be involved in inhibiting CTL lysis. CTL lysis of infected target cells involves a number of crucial events including adhesion and specific recognition, signal transduction and delivery of the "lethal hit". It is conceivable that one or more of these events is inhibited by infection with HSV.

The mechanism of inhibition of CTL lytic function by FB infected with HSV-1 for 2 hours is not known. Since cell-to-cell contact of HSV-FB and CTL was required for inhibition and paraformaldehyde-fixed FB previously infected for 2 hours inhibited CTL lytic activity (Posavad, unpublished observations), a viral or cellular protein expressed on the surface of the infected FB may mediate the inhibition. This protein could send a signal to the CTL via a membrane bound protein, to inactivate, or anergize the CTL. Interestingly, NK and LAK cells are not inhibited when exposed to FB infected with HSV-1 for 4 hours; inhibition of lytic function does not occur until FB are infected for at least 8 hours with HSV-1 (Confer et al., 1990). These findings suggest that inhibitory ability of FB infected

with HSV for 2 hours is specific for CTL and may involve a protein expressed on CTL but not on NK or LAK cells. It is more advantageous for a virus, such as HSV, to inhibit CTL lytic activity early in its replication cycle, allowing a productive infection to occur with the concommittant release of progeny virus. CTL lysis of cells during late times of infection would not be as important because progeny virus is already produced and the cell would eventually die when infectious virus was released.

HSV-infected LCL are the most common target cells used in vitro for human anti-HSV CTL. HSV can infect LCL, express viral proteins, and eventually the cells will die. LCL infected with HSV for 6 and 20 hours can equally serve as target cells unlike FB infected with HSV which do not serve as targets at any time-point. One explanation for this has been forwarded (Koelle et al., 1993) and suggests that LCL may be more efficient targets for CTL than FB because EBV transformation of B cells increases the density and number of cell surface molecules (HLA class I, LFA-3, ICAM-1) that are involved in effector-target interactions (McCune et al., 1975; Billaud et al., 1990). Alternatively, HSV-LCL may not inhibit CTL lytic activity since viral production is lower than from HSV-FB. Therefore the level of infectious virus required to inhibit CTL lytic function may not be achieved. This may explain why LCL infected for 20 hours are sensitive to lysis by CTL. However, at early times in the HSV replication cycle when infectious virus is not required to inhibit CTL-mediated lysis by HSV-FB, a protein expressed in HSV-FB, and not in HSV-infected LCL, may inhibit CTL lysis. Thus, it is not clear

if HSV-infected LCL are killed by CTL because they are better CTL targets than HSV-FB or because they are unable to inhibit CTL lytic function similar to HSV-FB. Replacing HSV-FB with HSV-infected LCL in a sandwich assay would determine their effect, if any, on CTL lytic function.

It is evident that the resistance of HSV-FB to lysis by CTL may be distinct from the ability of HSV-FB to inhibit CTL lysis. A recent study has demonstrated that the resistance of HSV-FB to lysis by human CTL clones may involve a late gene product and a decrease in the cell surface expression of HLA class I/β2-microglobulin leading to a blockade of antigen presentation (Koelle et al., 1993). Although the present study observed the resistance of HSV-FB to CTL lysis, the effect of exposure of CTL to HSV-FB was determined and not the mechanism of resistance. The resistance of HSV-FB to lysis by CTL may allow the virus to evade immune destruction which may be augmented by the ability of HSV-FB to suppress the lytic function of CTL.

F. Is the inhibition of CTL activity by HSV-FB relevant in vivo?

The inhibition of human CTL lytic function by HSV-FB or by direct infection with cell-free virus may have important implications in HSV infections. Immunosuppressed patients with defects in cell-mediated immunity can experience severe HSV infections (Rinaldo, 1990). Further, several markers of cell-mediated immune functions are depressed during recrudescence as compared to convalescence (Kuo and Lin, 1990; Vestey et al., 1989). These studies have focussed on systemic cell-mediated immune functions and may not reflect events

occurring within a herpetic lesion. Alos, it is not clear whether immunosuppression leads to reactivation of HSV or whether HSV reactivation results in immunosuppression. The results of the present study suggest that reactivation of latent HSV and subsequent infection of cells within a herpetic lesion may lead to a transient immunosuppression allowing the virus to spread to other cells. Perhaps CTL have varying sensitivities to the inhibitory effects of HSV infection and thus, CTL that are insensitive to the inhibitory effects lyse HSV-infected cells. In support of this, cloned 3G5 cells appear to be less sensitive to the inhibitory effects of HSV-FB and to inactivation by direct HSV infection (Posavad, unpublished observations). However, this cloned CTL line by not be representative of CTL in vivo. HSV-induced immunosuppression via infection of CTL, NK cells (Confer et al., 1990) and monocytes (Hayward et al., 1993) may account for the recurrent episodes and persistence of HSV in immunocompetant individuals.

G. Are CTL important in controlling HSV infections?

Although the results of many studies in humans and mice point to a crucial role of T cells in HSV infections, the involvement of T cells, especially CTL, in the control of HSV infections and reactivations has not been directly proven. Immunosuppressed patients, such as AIDS patients and transplant patients, exhibit severe and often life-threatening HSV infections. Adoptive transfer of murine $CD4^+$ or $CD8^+$ HSV-immune T cells protects naive mice from subsequent lethal challenge with HSV (Nash et al., 1987). The in vitro inhibition of CTL lytic function by HSV-infected FB and keratinocytes suggests that CTL within a herpetic lesion that

contact HSV-infected cells are unable to lyse these cells thereby permitting the spread of HSV. If this phenomenon occurs in vivo, then how are CTL involved in the control and resolution of herpetic lesions? Although CTL are presumed to control and clear HSV infections by specific lysis of infected cells, no in vivo evidence is available to support this theory. Functions other than direct cell lysis have been ascribed to CTL such as cytokine release, attraction of phagocytic cells, or causing lethal metabolic changes in the infected cell (Martin and Rouse, 1990). Interestingly, Martz and Howell (1989) proposed a theory of pre-lytic halt of viral replication by CTL as a mechanism of control of virus infections. The conventional lytic halt by CTL requires that CTL lyse targets rapidly by attacking the membrane of infected cells, a process detected using standard 5 hour ^{51}Cr-release assays. The pre-lytic halt hypothesis proposes that CTL halt viral replication by inducing internal disintegration without the neccessity to rapidly lyse target cells. Internal disintegration leads to eventual target cell death and prevention of release of viral progeny. In support of this, it has been demonstrated that CTL can induce apoptosis, or programmed cell death, in target cells involving the induction of a cellular endonuclease that causes DNA fragmentation (Ucker, 1987; Nishioka and Welsh, 1992). Recently, CTL have been shown to cause a pre-lytic halt of HSV replication in HSV-FB (Martz and Gamble, 1992). Therefore, the involvement of CTL in HSV infections may be through their ability to induce degradation of viral genomes prior to the production of progeny virus. Although HSV-FB inhibit the lytic function of CTL, it is not known if HSV-FB inhibit the ability of CTL to induce

pre-lytic halt of HSV replication in infected cells.

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APPENDICES

Appendix 1. Raw Data from Chapter 2, Table 1[a]

Donor	E:T	Virus Used To Infect LCL					
		Un	HSV	Allo-HSV	VVNP	VgB11	VgD52
KL	50:1	1[b]	34	6	5	33	35
	25:1	2	18	1	3	26	25
	12:1	0	8	0	2	16	18
GS	60:1	2	46	3	2	1	6
	30:1	2	36	1	1	2	5
	15:1	0	19	1	0	0	2
BL	30:1	5	29	10	11	12	11
	15:1	2	17	8	9	8	10
	7:1	1	12	4	6	5	4
TH	30:1	5	19	4	0	5	6
	15:1	6	12	2	2	4	2
	7:1	4	10	2	1	1	3
MW	50:1	0	15	2	4	2	0
	25:1	0	11	1	1	0	0
	12:1	0	9	0	1	0	0
CP	60:1	5	21	3	2	0	4
	30:1	1	16	0	0	0	2
	15:1	1	12	1	2	0	2

[a] PBMC were stimulated for 7 days with HSV-1 followed by use in a ^{51}Cr release assay with target cells (LCL) infected with HSV-1 (HSV), VVNP, VgB11, VgD52, or uninfected LCL (Un) or allogeneic LCL infected with HSV-1 (allo-HSV).

[b] Results shown are % specific ^{51}Cr release.

Appendix 2. Raw Data From Chapter 2, Table 2.[a]

Infected LCL Target	Undepleted	Cell Population Depleted		
		CD16/CD56	CD16/CD56 CD4	CD16/CD56 CD8
Un	9	6	2	4
HSV-1	45	41	17	27
Allo-HSV	18	9	6	5
VVNP	12	7	3	4
VgB11	32	27	12	16
VgD52	25	19	10	9

[a] Polyclonal cultures from KL were left undepleted or were depleted of CD16 and CD56 positive cells and CD4 or CD8 positive cells just prior to use in a ^{51}Cr release assay. Target cells were autologous LCL that were uninfected (Un) or infected with HSV-1, VVNP, VgB11 or VgD52 or allogeneic HSV-infected LCL (allo-HSV). Results shown are % specific ^{51}Cr release at an E:T ratio of 60:1

Appendix 3. Raw Data From Chapter 3, Figure 3.[a]

Infected LCL Target	Time of Infection (hr)	%Specific ^{51}Cr Release (±SD)
Mock	.....	6 (1)
HSV-1	24	35 (4)
VVNP	24	39 (2)
VgB11	24	34 (6)
VgD52	24	32 (4)
AdlE3	24	3 (3)
AdgB	24	7 (1)
AdgD	24	6 (0.5)
AdlE3	48	4 (1)
AdgB	48	5 (0.5)
AdgD	48	6 (2)
Allo HSV-1	24	7 (1)

[a] PBMC from KL were stimulated with HSV-1 for 7 days and tested for lytic activity against autologous LCL that were mock infected or infected with various viruses for various times, or allogeneic LCL infected with HSV-1 (allo-HSV). E:T ratio was 60:1.